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PATENT
Docket No. 290.0033 0101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Nancy W.Y. Ho et al.	Group Art Unit:	1653
Serial No.:	09/180,340	Examiner:	Hope A. Robinson
Confirmation No.:	6674		
Filed:	20 August 1999		

For: STABLE RECOMBINANT YEASTS FOR FERMENTING XYLOSE TO ETHANOL

Assistant Commissioner for Patents
Mail Stop Appeal Brief - Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANT'S BRIEF ON APPEAL

Sir:

This Brief is presented in support of the Appeal filed April 2, 2004, from the final rejection of claims 1-34 of the above-identified application under 37 C.F.R. §§1.113 and 1.191.

This Brief is being submitted in triplicate, as set forth in 37 C.F.R. § 1.192(a).

I. REAL PARTY IN INTEREST

The real party in interest of the above-identified application is the assignee, Purdue Research Foundation.

II. RELATED APPEALS AND INTERFERENCES

There are no appeals or interferences known to Appellant's Representatives which would directly affect, be directly affected by, or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 14-18, 28-30, and 32-34 are pending and are the subject of this Appeal (see Appendix A). Claims 1-13, 19-27, and 31 are canceled in an Amendment under 37 CFR §1.116 submitted concurrently with the present appeal brief.

IV. STATUS OF AMENDMENTS

A statement of the status of any amendment filed subsequent to the final rejection mailed October 2, 2003, is listed below.

A Final Office Action was mailed October 2, 2003. Claims 22 through 28 were rejected under 35 U.S.C. §112, second paragraph. Claims 1-13, 23-29, 31, and 34 were rejected under 35 U.S.C. §103(a) as being unpatentable over Ho et al. (WO95/13362) in view of Hallborn et al. (Canadian Patent No. 2,090,122). Claims 1-34 under were rejected under 35 U.S.C. 103(a) as obvious over Ho et al. (WO 95/13362) in view of Lopes et al. (*Yeast* 1996;12(5):467-77).

A Response under 37 CFR §1.116 was submitted to the Office on January 27, 2004. Claims 1, 10, 12, 14, 16, 18, 19, 23, 25, 26, 28, and 30 were amended, claims 20, 22, and 31 were canceled.

An Advisory Action was mailed February 27, 2004. The Advisory Action indicated the Response submitted to the Office on January 27, 2004 was not entered.

A second Response under 37 CFR §1.116 was submitted to the Office on April 2, 2004. Claims 14, 18, 21, 28, and 30 were amended, and claims 1-13, 20, 22-27, and 31 were canceled.

A second Advisory Action was mailed February 27, 2004. The Advisory Action indicated the Response submitted to the Office on February 27, 2004 was not entered.

An Amendment under 37 CFR §1.116 is submitted concurrently with the present appeal brief. The Amendment cancels claims 1-13, 19-27, and 31, and amends claims 28, 29, and 34. It is Appellant's understanding that this Amendment will be entered, as it cancels claims, adopts a suggestion by the Examiner (claim 28), and requires only cursory review by the Examiner (claims 28, 29, and 34). The claims involved in the present appeal (Appendix A) reflect the entry of the Amendment under 37 CFR §1.116 that is submitted concurrently with the present appeal brief.

V. SUMMARY OF THE INVENTION

The present invention provides methods for integrating multiple copies of exogenous DNA into reiterated chromosomal DNA of cells. The method includes replicating cells having reiterated genomic DNA and which contain a replicative and integrative plasmid containing the exogenous DNA to produce multiple generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA (specification, page 12, lines 6-17). In contrast to prior-reported methods for making yeasts with high copy number integrants, integration of an exogenous DNA by the present methods continues to take place gradually, long after transformation has been completed (specification, page 19, lines 15-22).

The present invention also provides plasmid vectors for integrating an exogenous DNA sequence including a selection marker into chromosomal DNA of a target yeast cell. The plasmid vectors contain an autonomous replicating sequence (specification, page 18, lines 27-31, and page 19, lines 13-15). The plasmid vectors include an exogenous DNA including the selection marker flanked on each end by a DNA flaking sequence which is homologous to a reiterated ribosomal DNA sequence of the target yeast cell (specification, page 11, lines 16-24).

VI. ISSUES PRESENTED FOR REVIEW

Issue A. Whether the Examiner has presented a *prima facie* case of obviousness under 35 U.S.C. 103(a) for claims 14-18, 28-30, and 32-34 as being unpatentable over Ho et al. (WO 95/13362) in view of Lopes et al. (*Yeast* 1996;12(5):467-77).

Issue B. Whether the Examiner has presented a *prima facie* case of obviousness under 35 U.S.C. 103(a) for claims 28, 29, and 34 as being unpatentable over Ho et al. (WO 95/13362) in view of Hallborn et al. (Canadian Patent No. 2,090,122).

VII. GROUPING OF CLAIMS

With respect to Issue A, for the purposes of this appeal claims 14-16, 18, 30, and 32-33 stand or fall together. Claims 28 and 34 stand or fall together. Claims 17 and 29 stand or fall together.

With respect to Issue B, for the purposes of this appeal claims 28, 29, and 34 stand or fall together.

In compliance with 37 C.F.R. §1.192(c)(7), the reasons the different groups of claims are separately patentable are presented in the argument section of this Brief.

VIII. ARGUMENT

ISSUE A. THE EXAMINER HAS FAILED TO ESTABLISH THE PRESENCE OF ALL THE CLAIM LIMITATIONS WHEN THE CITED DOCUMENTS ARE COMBINED, AND THE NECESSARY MOTIVATION FOR COMBINING THE CITED DOCUMENTS.

Claims 14-19, 28-30, and 32-34 stand rejected under 35 U.S.C. §103(a) over Ho et al. (WO95/13362) in view of Lopes et al. (*Yeast* 12(5), 467-77 (1996)). Appellants respectfully disagree, and request review and reversal of this rejection by the Board.

"To establish a prima facie case of obviousness . . . [f]irst, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the...references when combined...must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure." M.P.E.P. §706.02(j). *See also, In re Vaeck*, 20 USPQ2d 1438,1442 (Fed. Cir. 1991).

Claims 14-16, 18, 30, and 32-33

A document relied upon to support the present rejection is not positively included in a statement of the rejection

The Final Office dated October 2, 2003 asserts at page 8, first paragraph, that Lopes et al. disclose that "[m]ultiple copies of the plasmid were successfully integrated into the genome (over 140 copies); which are stably maintained in non-selective medium for multiple generations over long periods of time." This appears to refer to a statement in Lopes et al. at page 467, column 2, but it does not appear to refer to the experimental work described in Lopes et al.; this statement in Lopes et al. cites another document. "Where a reference is relied on to support a rejection, whether or not in a minor capacity, that reference should be positively included in the statement of the rejection." M.P.E.P. §706.02(j). *See also, In re Hoch*, 166 USPQ 406, 407 n. 3 (CCPA 1970). Since this document cited in Lopes et al. is relied upon to support the present rejection, and the document is not positively included in a statement of the rejection, it is respectfully submitted that this statement should not be afforded any weight by the Board.

The cited documents do not teach or suggest all the claim limitations when combined

Independent claims 14 and 18 recite a method including transforming cells with a "replicative and integrative plasmid comprising an autonomous replicating sequence" and exogenous DNA, and repeatedly replicating the cells to "produce progeny cells having multiple integrated copies of the exogenous DNA" (claim 14) or to "result in progeny cells each containing multiple integrated copies of the exogenous DNA" (claim 18). Independent claim 30 recites a method including replicating cells containing a "replicative and integrative plasmid comprising an autonomous replicating sequence and containing the exogenous DNA" to "produce progeny cells having multiple integrated copies of the exogenous DNA." The cited art does not teach or suggest such a method. Specifically, the cited prior art does not teach or suggest the use of a replicative and integrative plasmid comprising an autonomous replicating

sequence and exogenous DNA in a cell to result in progeny of the cell having multiple integrated copies of the exogenous DNA.

Ho et al. disclose the use of plasmids containing the 2-μm origin of replication (see Ho et al. at page 15, line 29 through page 16, line 31). It is well known in the art that there are several distinct types of chimeric plasmid vectors, including "(i) YIp (yeast integrating plasmids), which are unable to replicate and transform by integration into the genome of the recipient strain; (ii) YEp (yeast episomal plasmids), which carry the replication origin of the yeast 2-μm circle, an endogenous yeast plasmid, and can replicate in the recipient cell; and (iii) YRp (yeast replicating plasmids), which can replicate utilizing yeast autonomous replicating sequences (ARS)" (Gietz et al., BioTechniques, 30, 816-831 (2001), at page 817, col. 2 [1]). Thus, a 2-μm origin of replication and a yeast autonomous replicating sequence origin of replication are recognized in the art as different replication origins. Ho et al. teach that the 2-μm origin of replication permits the plasmid to replicate, but not integrate, in S. cerevisiae and closely related species (Ho et al., page 16, lines 1-4 and 26-31). The plasmids of Ho et al. are able to replicate, but they do not integrate. Thus, they are not replicative and integrative plasmids. Accordingly, Ho et al. do not teach or suggest a method including the use of a replicative and integrative plasmid comprising an autonomous replicating sequence.

Lopes et al. teach two types of plasmid vectors, pMIRY1 and pMIRY2, and other plasmids based on pMIRY1 and pMIRY2. The pMIRY1-based plasmid vectors include yeast rDNA, a synthetic oligonucleotide, the LEU2d gene, and pUC9 sequences (see Lopes et al. at page 468, column 2). The pMIRY2-based plasmid vectors include yeast rDNA, a chloroplast DNA marker, the LEU2d gene, and pBR322 sequences (see Lopes et al. at page 468, column 1). The plasmid vectors of Lopes et al. permit replication in E. coli, but the plasmid vectors do not include an autonomous replicating sequence that permits both replication and integration in the same cell. Accordingly, Lopes et al. do not teach or suggest a method including the use of a replicative and integrative plasmid comprising an autonomous replicating sequence.

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[1] While Gietz et al. was published after the filing date of the present application, the quoted passage references a document published in 1979.

Since neither Ho et al. nor Lopes et al. teach or suggest a method including the use of a replicative and integrative plasmid comprising an autonomous replicating sequence, combining Ho et al. and Lopes et al. could not result in a method including transforming cells with a "replicative and integrative plasmid comprising an autonomous replicating sequence" and exogenous DNA, and repeatedly replicating the cells to "produce progeny cells having multiple integrated copies of the exogenous DNA" (claim 14) or to "result in progeny cells each containing multiple integrated copies of the exogenous DNA" (claim 18). Combining Ho et al. and Lopes et al. could not result in a method including replicating cells containing a "replicative and integrative plasmid comprising an autonomous replicating sequence and containing the exogenous DNA" to "produce progeny cells having multiple integrated copies of the exogenous DNA" (claim 30). Accordingly, when combined the cited documents do not teach or suggest all the claim limitations of independent claims 14-18 and 30. Thus, the Examiner has failed to present a *prima facie* case that independent claims 14-18 and 30 are obvious in view of the cited art.

There is no motivation to combine the cited documents

"To support the conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references." M.P.E.P. §706.02(j). *See also, Ex parte Clapp,* 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985).

The Final Office Action dated October 2, 2003 states "it would have been obvious . . . to arrive at the claimed invention as a whole . . . because Ho et al. teach [] the simultaneous fermentation of xylose and glucose into ethanol from the yeast Saccharomyces cerevisiae . . . and Lopes et al. teach a method of making transformants stably maintained in non-selective medium for multiple generations over long periods of time" (Final Office Action, page 8, first full paragraph). The Final Office Action dated October 2, 2003 also states "[o]ne of ordinary skill in the art would be motivated to combine the teaching of both references because the method taught by Ho et al. introduces DNA into the same yeast taught by Lopes et al." (Final

Office Action, page 8, first full paragraph). This statement may show that Ho et al. and Lopes et al. could be combined; however, it does not provide any convincing line of reasoning as to why a skilled person would combine the documents. "The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." M.P.E.P. §2143.01 (emphasis in original). *See also, In re Mills,* 16 USPQ2d 1430, 1432 (Fed Cir. 1990). It is respectfully submitted that the use of the same yeast in each cited document does not provide any suggestion as to the desirability of making the combination. Thus, the Examiner has not provided any motivation to combine the cited documents, and therefore the Examiner has failed to present a *prima facie* case that claims 14-16, 18, 28-30, and 34 are obvious in view of the cited art.

Claims 28 and 34

In addition to the comments above regarding claims 14-16, 18, 30, and 32-33, the Board is also requested to consider the following comments when reviewing the patentability of claims 28 and 34.

The cited documents do not teach or suggest all the claim limitations when combined

Claims 28 and 34 recite a "plasmid vector comprising a functional yeast autonomous replicating sequence." The cited art does not teach or suggest such a plasmid.

Ho et al. disclose the use of plasmids containing a 2-μm origin of replication and, as discussed above, a 2-μm origin of replication and a yeast autonomous replicating sequence origin of replication are recognized in the art as different replication origins. Thus, the 2-μm origin of replication is not an autonomous replicating sequence. Ho et al. teach the use of plasmids containing the 2-μm origin of replication, but do not teach or suggest the use of a plasmid with a yeast autonomous replicating sequence as an origin of replication.

Lopes et al. teach two types of plasmid vectors, pMIRY1 and pMIRY2, and other plasmids based on pMIRY1 and pMIRY2. The pMIRY1-based plasmid vectors include yeast rDNA, a synthetic oligonucleotide, the LEU2d gene, and pUC9 sequences (see Lopes et al. at

page 468, column 2). The pMIRY2-based plasmid vectors include yeast rDNA, a chloroplast DNA marker, the LEU2d gene, and pBR322 sequences (see Lopes et al. at page 468, column 1). The plasmid vectors of Lopes et al. do not include a yeast autonomous replicating sequence, nor do Lopes et al. suggest the use of a yeast autonomous replicating sequence. Thus, Lopes et al. do not teach or suggest a "plasmid vector comprising a functional yeast autonomous replicating sequence."

Since neither Ho et al. nor Lopes et al. teach or suggest a "plasmid vector comprising a functional yeast autonomous replicating sequence," combining Ho et al. and Lopes et al. could not result in a plasmid having a yeast autonomous replicating sequence. Accordingly, when combined the cited documents do not teach or suggest all the claim limitations of independent claims 28 and 34. Thus, the Examiner has failed to present a *prima facie* case that claims 28 and 34 are obvious in view of the cited art.

Claims 17 and 29

In addition to the comments above regarding claims 14-16, 18, 30, and 32-33, and claims 28 and 34, the Board is also requested to consider the following comments when reviewing the patentability of claims 17 and 29.

There is no motivation to combine the cited documents

Claim 17 is dependent upon claim 14, which recites a method including "transforming the cells with a replicative and integrative plasmid comprising an autonomous replicating sequence [and] exogenous DNA . . . ." Claim 17 recites "wherein . . . the exogenous DNA includes genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase." Independent claim 29 recites a "plasmid vector comprising . . . exogenous DNA including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase."

The Final Office Action dated October 2, 2003 asserts that it "would have been obvious to . . . arrive at the claimed invention as a whole by combining the teachings of Ho et al. and Lopes et al. because . . . Lopes teach a method of making transformants stably maintained in non-selective medium for multiple generations over long periods of time" (Final Office Action,

page 8, first full paragraph). Thus, for motivation, the Examiner relies upon Lopes' teaching of making stable transformants. However, the Examiner's statement is not true when the DNA to be inserted includes the genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase (dependent claim 17 and independent claim 29). Specifically, when a DNA fragment of Ho et al. (i.e., a DNA fragment including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase) is combined with a plasmid of Lopes et al., the skilled person would not expect the method of Lopes et al. to result in stable transformants that are maintained in non-selective medium. The reasons for this are discussed immediately below.

Lopes et al. state that "[i]n this paper we describe studies aimed at establishing whether pMIRY-type plasmids can indeed be stably maintained under the conditions applied during industrial production of proteins" (see Lopes et al. at page 467, col. 2, last sentence). The authors go on to clearly prove that the pMIRY-type plasmids are not stably maintained under certain conditions. Lopes et al. disclose that "the mitotic stability of pMIRY2 vectors carrying such a [foreign] gene is decreased significantly" and yeast transformed with such a vector "lose some 80% of their vector copies over a period of 70 generations of growth in non-selective medium containing galactose as the sole carbon source" (see Lopes et al. page 472, col. 2). Lopes et al. further disclose that "plasmid size is a crucial factor in determining mitotic stability of the pMIRY-type vectors" (see Lopes et al. page 473, col. 2, first paragraph, lines 17-21), emphasis added). Moreover, the Board is requested to consider with particularity that Lopes et al. state that "[s]table maintenance is only observed when the complete plasmid has a size no larger than that of the rDNA unit (9.1 kb)" (abstract, emphasis added, also see page 473, col. 2, first paragraph, for a similar statement). This is notable because adding the exogenous DNA of Ho et al., i.e., the genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, to the smallest plasmid taught by Lopes et al. would result in a plasmid with a size greater than 9.1 kb. The reasoning showing how such a plasmid would be greater than 9.1 kb is presented in the following paragraph.

The smallest pMIRY-type vector disclosed by Lopes et al. is pMIRY1, which has a size of about 6.1 kb (see Lopes et al. at the bottom of Figure 1B). The DNA fragment disclosed by Ho et al. that includes the genes encoding xylose reductase, xylitol dehydrogenase, and

xylulokinase is at least 4.9 kb in size.[2] The addition of the Ho et al. DNA fragment to even the smallest pMIRY-type vector would result in a vector of at least 11 kb. This is greater than the size limit of 9.1 kb disclosed by Lopes et al., and thus will result in a vector having reduced mitotic stability.

Thus, the Examiner asserts that the skilled person would be motivated to combine the two cited documents "because...Lopes teach a method of making transformants stably maintained in non-selective medium for multiple generations over long periods of time." (Final Office Action, page 8, first full paragraph). However, this assertion is false when Ho et al. and Lopes et al. are combined, because the DNA to be inserted includes the genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase. Since the motivation for combining the cited documents is based on a false premise, i.e., that the resulting transformants will be stably maintained in non-selective medium for multiple generations, the Examiner has not provided any motivation to combine the cited documents. Therefore, the Examiner has failed to present a *prima facie* case that claims 17 and 29 are obvious in view of the cited art.

Summary

For at least the above reasons, Appellant respectfully submits that the rejection of claims 14-16, 18, 28-30, and 32-34 does not meet the requirements for a *prima facie* case of obviousness. Review and reversal by the Board of the rejection of claims 14-16, 18, 28-30, and 32-34 are, therefore, respectfully requested.

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[2]Ho et al. disclose that the size of the translated region of the xylulokinase gene is 2.1 kb (see Example 3, page 22, line 25-26). Ho et al. also disclose at Example 2 that the size of the xylitol dehydrogenase gene and its associated promoter are 1.9 kb and 910 bp, respectively (see page 22, lines 11-14). This is a total of 4.9 kb, and does not include the xylose reductase gene.

ISSUE B. THE EXAMINER HAS FAILED TO ESTABLISH THE PRESENCE OF ALL THE CLAIM LIMITATIONS WHEN THE CITED DOCUMENTS ARE COMBINED, THE NECESSARY MOTIVATION FOR COMBINING THE CITED DOCUMENTS, AND A REASONABLE EXPECTATION OF SUCCESS.

Claims 28, 29, and 34 stand rejected under 35 U.S.C. §103(a) over Ho et al. (WO95/13362) in view of Hallborn et al. (Canadian Patent No. 2,090,122). Appellants respectfully disagree, and request review and reversal of this rejection by the Board.

"To establish a prima facie case of obviousness . . . [f]irst, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the...references when combined...must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure." M.P.E.P. §706.02(j). *See also, In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438,1442 (Fed. Cir. 1991).

The cited documents do not teach or suggest all the claim limitations when combined

Claim 28 recites a "plasmid vector comprising a functional yeast autonomous replicating sequence and an exogenous DNA . . . flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell." Claim 29 recites a "plasmid vector comprising a functional yeast autonomous replicating sequence and exogenous DNA . . . flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of a target yeast cell." Claim 34 recites a "plasmid vector comprising a functional yeast autonomous replicating sequence and exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell." The cited art does not teach or suggest a plasmid having these components.

Ho et al. disclose the use of plasmids containing a 2-μm origin of replication and, as

discussed above, a 2-μm origin of replication and a yeast autonomous replicating sequence origin of replication are recognized in the art as different replication origins. Thus, the 2-μm origin of replication is not an autonomous replicating sequence. Ho et al. do not teach or suggest the use of a plasmid having a yeast autonomous replicating sequence as an origin of replication and exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell.

As discussed in greater detail immediately below, some vectors taught by Hallborn et al. include exogenous DNA and are capable of replicating autonomously, however, these vectors do not have exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of a target yeast cell. Other vectors taught by Hallborn et al. include exogenous DNA flanked on each end by DNA homologous to a reiterated DNA sequence, but these vectors do not have a "functional yeast autonomous replicating sequence " (claims 28, 29, and 34).

Vectors taught by Hallborn et al. include a multicopy or single copy vector "capable of replicating autonomously when transformed into the recipient yeast strain"(see Hallborn et al., page 7, lines 10-17, see also the paragraph spanning pages 10 and 11). As disclosed by Hallborn et al., the vectors capable of replicating autonomously include an exogenous DNA, but such vectors do not include flanking DNA that is homologous to a reiterated DNA sequence of a target yeast cell. Furthermore, Hallborn et al. do not teach or suggest the use of flanking DNA in this type of vector. Thus, the combination of the vectors of Ho et al. with this type of vector of Hallborn et al. would result in a vector containing exogenous DNA and an origin of replication that permits autonomous replication, but the exogenous DNA would not be flanked by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell.

Hallborn et al. also teach vectors for integrating genes into the yeast genome, for instance, at a ribosomal RNA locus (see Hallborn et al. at, for instance, page 7, lines 21-34, and example 5 at page 17, lines 16-30). An example of such a vector is disclosed at Figure 5, and includes an exogenous DNA ("XR DNA" in Figure 5 of Hallborn et al.) flanked on each end by a ribosomal DNA sequence. However, Hallborn et al. teach that such vectors are made using the

BS+ vector (see Hallborn et al. at page 7, lines 21-26), a well known vector containing a ColE1 origin of replication for use in E. coli, but not containing an origin of replication that functions in yeast. Furthermore, Hallborn et al. do not teach or suggest the use of a functional yeast autonomous replicating sequence in this type of vector. Thus, the combination of the vectors of Ho et al. with this type of vector of Hallborn et al. would result in a vector containing a 2-μm origin of replication, exogenous DNA, and flanking DNA that is homologous to a reiterated ribosomal DNA sequence of a target yeast cell, but would not contain a functional yeast autonomous replicating sequence (claims 28, 29, and 34).

The Final Office Action dated October 2, 2003 further asserts that Hallborn et al. teach "a method of transforming cells with integrative plasmids" (see Final Office Action at page 5, first full paragraph, last line). This is not correct. Hallborn et al. teach the construction of a plasmid carrying an exogenous DNA that is to be integrated, but the exogenous DNA, flanked by ribosomal sequences, is released and the resulting non-replicating fragment is cotransformed into a yeast with an autonomously replicating plasmid that does not integrate (see Hallborn et al., page 7, lines 26-29, and example 5 at page 17, lines 22-25). Thus, Hallborn et al. teach a method of transforming cells with an integrative fragment and a non-integrative plasmid, not an integrative plasmid.

The Final Office Action dated October 2, 2003 also asserts that inconsistent and contradictory arguments were presented regarding the assertion that none of the cited documents teach the product plasmid vector, and refers to Appellant's statement that "Hallborn et al. teach integration of foreign DNA into the yeast genome . . . An autonomously replicating plasmid is also introduced" (Final Office Action, page 6, first full paragraph). As argued immediately below, the autonomously replicating plasmid taught by Hallborn et al. does not include "exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell" (claims 28 and 34), or "exogenous DNA . . . flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of a target yeast cell" (claim 29).

When Hallborn et al. teach integrating genes into the yeast genome, they teach the introduction of two separate DNAs; the first is a non-replicating DNA fragment that is

integrated, the second is an autonomously replicating plasmid that does not integrate. The autonomously replicating plasmid does not contain any exogenous DNA that is "flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell" (claims 28 and 34), or "flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of a target yeast cell" (claim 29). The purpose of the autonomously replicating plasmid is to permit identification of transformants, and it is later removed from the cells (see Hallborn et al. at page 7, lines 27-31, and Example 5 at page 17, lines 24-30). Thus, while Hallborn et al. may teach the introduction of an autonomously replicating plasmid when genes are to be integrated into a yeast genome, the autonomously replicating plasmid does not contain any exogenous DNA that is flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of a target yeast cell.

Since neither Ho et al. nor Hallborn et al. teach or suggest a "plasmid vector comprising a functional yeast autonomous replicating sequence and an exogenous DNA . . . flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell" (claim 28), a "plasmid vector comprising a functional yeast autonomous replicating sequence and exogenous DNA . . . flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of a target yeast cell" (claim 29), or "plasmid vector comprising a functional yeast autonomous replicating sequence and exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell" (claim 34). Accordingly, when combined the cited documents do not teach or suggest all the claim limitations of independent claims 28, 29, and 34. Thus, the Examiner has failed to present a *prima facie* case that claims 28, 29 and 34 are obvious in view of the cited art.

There is no motivation to combine or modify the cited documents

To support the conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the

claimed invention to have been obvious in light of the teachings of the references." M.P.E.P. §706.02(j). *See also, Ex parte Clapp,* 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985).

The Final Office Action dated October 2, 2003 states "it would have been obvious to one of ordinary skill in the art to arrive at the claimed invention as a whole because Ho et al. and Hallborn et al. teach the fermentation of sugars to ethanol (i.e., xylose to ethanol) using the same strain of yeast" (Final Office Action, page 5, last paragraph). This statement may show that the two documents could be combined; however, it does not provide any convincing line of reasoning as to why a skilled person would combine the documents. "The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." M.P.E.P. §2143.01 (emphasis in original). *See also, In re Mills,* 16 USPQ2d 1430,1432 (Fed Cir. 1990). It is respectfully submitted that the use of the same yeast in each cited document does not provide any suggestion as to the desirability of making the combination. Thus, the Examiner has not provided any motivation to combine the cited documents.

The Final Office Action dated October 2, 2003 states "one of skill would be motivated to combine the teachings of the references because Ho et al. disclose that ethanol is an ideal liquid fuel for automobiles and Hallborn et al. disclose a method to perform stable transformation over time" (Final Office Action, paragraph bridging pages 5 and 6). Hallborn et al. does not appear to make any statements regarding the stability of the vectors used to transform yeast. Moreover, the Examiner has not provided any reasoning as to why such vectors would be expected to be stable. Accordingly, there is no basis in Hallborn et al. for the Examiner's statement that "Hallborn et al. disclose a method to perform stable transformation over time." Since Hallborn et al. is silent regarding the stability of the transformations, the asserted stability cannot be used by the Examiner to argue that the skilled worker would be motivated to combine or modify the documents. Thus, the Examiner has not provided any motivation to combine the cited documents.

There is no reasonable expectation of success

The Final Office Action dated October 2, 2003 states "[o]ne of skill in the art would

reasonable [sic] expect successful results by combining the two references because Hallborn et al. teach integrative plasmids and an autonomously replicating plasmid suitable for carrying out transformation stably (Final Office Action, paragraph bridging pages 5 and 6).

As discussed above, Hallborn et al. teach non-replicative integrative fragments, and do not teach or suggest integrative plasmids. Briefly, Hallborn et al. teach the construction of a plasmid carrying an exogenous DNA that is to be integrated, but the exogenous DNA, flanked by ribosomal sequences, is released and the resulting non-replicative fragment is cotransformed into a yeast with an autonomously replicating plasmid (see Hallborn et al., page 7, lines 26-29). Thus, Hallborn et al. teach a method of transforming cells with an integrative fragment and a non-integrative plasmid, not an integrative plasmids.

The Examiner's conclusion that there is a reasonable expectation of success is based on the premise that Hallborn et al. teach integrative plasmids; however, this premise is false. Since the premise is false, the conclusion is necessarily false. Thus, the Examiner's assertion that there is a reasonable expectation of success is unfounded.

Summary

For at least the above reasons, Appellant respectfully submits that the rejection of claims 28, 29, and 34 do not meet the requirements for a *prima facie* case of obviousness. Review and reversal by the Board of the rejection of claims 28, 29, and 34 are, therefore, respectfully requested.

SUMMARY

For the foregoing reasons, Appellants respectfully submit that a *prima facie* case of obviousness has not been established for the pending claims. It is respectfully requested that the Board review and reverse the rejections of claims 14-19, 28-30, and 32-34, and that all the claims be allowed.

CERTIFICATE UNDER 37 C.F.R. 1.10:

The undersigned hereby certifies that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date indicated below and is addressed to the Assistant Commissioner for Patents, Mail Stop Appeal Brief - Patents, P.O. Box 1450, Alexandria, VA 22313-1450

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APPENDIX A - PENDING CLAIMS

Serial No.: 09/180,340

Docket No.: 290.00330101

14. A method of integrating multiple copies of exogenous DNA into reiterated chromosomal DNA of cells, comprising:

(a) transforming the cells with a replicative and integrative plasmid comprising an autonomous replicating sequence, exogenous DNA, and a first selection marker; and

(b) repeatedly replicating the cells from step (a) to produce a number of generations of progeny cells while selecting for cells which include the selection marker, promoting the retention of the replicative and integrative plasmid in subsequent generations of the progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA.

15. The method of claim 14, wherein the plasmid DNA also includes a second selection marker for selecting cells which include the plasmid.

16. The method of claim 14 wherein the cells are yeast or eukaryotic cells, and wherein the method further includes the step of repeatedly replicating the progeny cells from step (b) to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells and recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

17. The method of claim 16 wherein the cells are yeast cells and the exogenous DNA includes genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, which also serve as the first selection marker.

18. A method of integrating multiple copies of exogenous DNA into reiterated chromosomal DNA of cells, comprising:

(i) transforming yeast cells with a replicative and integrative plasmid comprising an autonomous replicating sequence, exogenous DNA, and a selection marker, the exogenous DNA being flanked on each end by a DNA sequence homologous to a reiterated sequence of DNA of the host;

(ii) repeatedly replicating the transformed yeast cells from step (i) to produce a number of generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative plasmid in subsequent generations of the progeny cells and result in progeny cells each containing multiple integrated copies of the exogenous DNA; and

(iii) replicating the progeny cells from step (ii) to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells and recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

28. A plasmid vector comprising a functional yeast autonomous replicating sequence and an exogenous DNA comprising a first selection marker, the exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell, the plasmid further including a second selection marker in a position other than between the DNA flanking sequences, the plasmid vector for use in integrating the exogenous DNA sequence into chromosomal DNA of the target yeast cell.

29. A plasmid vector comprising a functional yeast autonomous replicating sequence and exogenous DNA including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of a target yeast cell, the plasmid vector for use in integrating the exogenous DNA sequence into chromosomal DNA of the target yeast to form stable integrants

which ferment xylose to ethanol.

30. A method for producing cells having multiple integrated copies of an exogenous DNA fragment, comprising:

replicating cells having reiterated genomic DNA and which contain a replicative and integrative plasmid comprising an autonomous replicating sequence and containing the exogenous DNA to produce multiple generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA.

32. The method of claim 14 wherein the cells are yeast.

33. The method of claim 30 wherein the cells are yeast.

34. A plasmid vector comprising a functional yeast autonomous replicating sequence and exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of a target yeast cell, the plasmid further comprising a selection marker in a position other than between the DNA flanking sequences, the plasmid vector for use in integrating an exogenous DNA sequence into chromosomal DNA of the target yeast cell.

APPENDIX B - CITED AUTHORITIES AND DOCUMENTS

Serial No.: 09/180,340
Docket No.: 290.00330101

1. Ho et al. (International Publication No. WO 95/13362)
2. Hallborn et al. (Canadian Patent No. 2,090,122)
3. Lopes et al. *Yeast* 1996;*12*(5);467-77
4. M.P.E.P. §706.02(j), Eighth Edition, Revision 2(May 2004)
5. *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991)
6. *In re Hoch*, 166 USPQ 406 (CCPA 1970)
7. Gietz et al. *BioTechniques* 2001;*30*:816-831
8. *Ex Parte Clapp* 227 USPQ 972 (Bd. Pat. App. & Inter. 1985)
9. M.P.E.P. §2143.01, Eighth Edition, Revision 2(May 2004)
10. *In re Mills,* 16 USPQ2d 1430 (Fed Cir. 1990)

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(54) Title: RECOMBINANT YEASTS FOR EFFECTIVE FERMENTATION OF GLUCOSE AND XYLOSE

(57) Abstract

Described are recombinant yeasts containing genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase, and DNA molecules, vectors and methods useful for producing such yeasts. The recombinant yeasts effectively ferment xylose to ethanol, and preferred yeasts are capable of simultaneously fermenting glucose and xylose to ethanol thereby taking full advantage of these two sugar sources as they are found in agricultural biomass.

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RECOMBINANT YEASTS FOR EFFECTIVE FERMENTATION OF GLUCOSE AND XYLOSE

BACKGROUND OF THE INVENTION

The present invention relates generally to genetically engineered yeasts capable of simultaneously fermenting the two major sugar constituents of cellulosic biomass, glucose and xylose, to ethanol. More particularly, the present invention relates to such yeasts which can be constructed by cloning a xylose reductase gene, a xylitol dehydrogenase gene, and a xylulokinase gene in yeasts capable of fermenting glucose to ethanol.

Recent studies have proven ethanol to be an ideal liquid fuel for automobiles. It can be used directly as a neat fuel (100% ethanol) or as a blend with gasoline at various concentrations.

The use of ethanol to supplement or replace gasoline can reduce the dependency of many nations on imported foreign oil and also provide a renewable fuel for transportation. Furthermore, ethanol has proven a cleaner fuel that releases far less pollutants into the environment than regular gasoline. For example, it has

been demonstrated that the use of oxygenated materials in gasoline can reduce the emission of carbon monoxide, a harmful pollutant, into the air. Among the several oxygenates currently used for boosting the oxygen content of gasoline, ethanol has the highest oxygen content. The United States Environmental Protection Agency (EPA) has shown that gasoline blended with 10% ethanol reduces carbon monoxide emissions by about 25%-30%.

Up to now, the feedstock used for the production of industrial alcohol by fermentation has been sugars from sugar cane or beets, starch from corn or other food crops. However, these agricultural crops are too expensive to be used as feedstock for the large-scale production of fuel ethanol.

Plant biomass is an attractive feedstock for ethanol-fuel production by fermentation because it is renewable, and available at low cost and in large amounts. The concept of using alcohol produced by microbial fermentation of sugars from agricultural biomass had its nascense at least two decades ago. The major fermentable sugars from cellulosic materials are glucose and xylose (with the ratio of glucose to xylose being approximately 2 or 3 to 1). The most desirable fermentations of cellulosic materials would, of course, completely convert both glucose and xylose to ethanol. Unfortunately, even now there is not a single natural known microorganism capable of fermenting both glucose and xylose effectively.

Yeasts, particularly Saccharomyces, have traditionally been used for fermenting glucose-based feedstocks to ethanol, and they are still the best microorganisms for converting glucose to ethanol.

However, these glucose-fermenting yeasts have been found not only unable to ferment xylose but also unable to use the pentose sugar for growth. Nevertheless, these glucose-fermenting yeasts can use xylulose for growth and fermentation (Figure 1), albeit with varying efficacy. For example, S. cerevisiae ferments xylulose very poorly while species of Schizosaccharomyces does so quite effectively (Chiang et al., 1981; Lastick et al., 1989).

Even though the glucose-fermenting yeasts are unable to use xylose both for growth and fermentation, there are many natural yeasts that can use xylose for growth aerobically but they cannot ferment xylose to ethanol. These xylose-using/non-fermenting yeasts rely upon two enzymes--xylose reductase and xylitol dehydrogenase--to convert xylose to xylulose. These yeasts are different from most bacteria which rely on a single enzyme--xylose isomerase--to convert xylose directly to xylulose (Figure 1). The yeast xylose reductase and xylitol dehydrogenase also require cofactors for their actions; xylose reductase depends on NADPH as its cofactor and xylitol dehydrogenase depends on NAD as its cofactor. On the contrary, bacterial xylose isomerase requires no cofactor for direct conversion of xylose to xylulose (Figure 1).

Two decades ago, much effort was devoted in an attempt to find new yeasts capable of effectively fermenting both glucose and xylose to ethanol. Although no such ideal yeast has been found, those efforts did have limited success. For example, a few yeasts were found to be capable not only of utilizing xylose for growth aerobically, but also of fermenting xylose to ethanol (Toivola et al., 1984; Dupreez and vander Walt, 1983), although none of these xylose-fermenting yeasts were totally effective in fermenting xylose to ethanol

(Jeffries, 1985). In addition, these yeasts are unable to ferment glucose effectively.

Among the xylose-fermenting yeasts, three species, Pachysolen tannophilus (Toivola et al., 1984), Candida shehatae (Dupreez and van der Walt, 1983), and Pichia stipitis (Grootjen et al., 1990) have been extensively characterized. P. stipitis and C. shihatae ferment xylose better than other xylose-fermenting yeasts (Grootjen et al., 1990). Nevertheless, even the best xylose-fermenting yeasts lack high efficiency in fermenting xylose, and are also highly ineffective in fermenting glucose (Jeffries, 1985).

In the past decade, efforts were also made to genetically modify traditional glucose-fermenting yeasts, particularly S. cerevisiae, by recombinant DNA techniques. Initial efforts were concentrated on cloning a xylose isomerase gene into yeast to render it capable of converting xylose directly to xylulose without dependence on cofactors. However, these efforts have been unsuccessful because the genes encoding various bacterial xylose isomerases are incapable of directing the synthesis of an active enzyme in S. cerevisiae (Rosenfeld et al., 1984; Ho et al., 1983; Sarthy et al., 1987; Wilhelm and Hollenberg, 1984; Amore et al., 1989)).

In the last few years, efforts toward genetically engineering yeasts, particularly S. cerevisiae, to ferment xylose have been focused on cloning genes encoding xylose reductase (Takama et al., 1991; Hallborn et al., 1991; Strasser et al., 1990), xylitol dehydrogenase (Köetter et al., 1990; Hallborn et al., 1990), and xylulokinase (Stevis et al., 1987; Chang and Ho, 1988; Ho and Chang, 1989; Deng and Ho, 1990). S. cerevisiae and other

glucose-fermenting yeasts do not contain any detectable xylose reductase or xylitol dehydrogenase activities, but all seem to contain xylulokinase activity. Thus, the glucose-fermenting yeasts can all ferment xylulose, but do so with varying efficacy (Deng and Ho, 1990).

Recently, Köetter et al. (1990), Strasser et al. (1990), and Hallborn et al. (1990; 1991), have cloned both the xylose reductase and the xylitol dehydrogenase gene in S. cerevisiae. However, these genetically engineered yeasts still cannot effectively ferment xylose. For example, these yeasts have been incapable of fermenting more than 2% xylose. In addition, they produce large amounts of xylitol from xylose (Hallborn et al., 1990; Köetter and Ciriacy, 1993), which diverts the valuable xylose substrate from the desired fermentive path to ethanol.

The extensive background in this field as outlined above demonstrates that despite the concerted and longstanding efforts of numerous researchers, yeasts capable of effectively fermenting both glucose and xylose to ethanol have not been achieved. Accordingly, there remain needs for such yeasts and for methods of their preparation and use. It is to these needs that the present invention is addressed.

SUMMARY OF THE INVENTION

A feature of this invention relates to the discovery that new yeast strains capable of effectively fermenting xylose alone or simultaneously with glucose can be created using recombinant DNA and gene cloning techniques. Particularly, these techniques have been used to create new recombinant yeasts containing cloned xylose reductase (XR), xylitol dehydrogenase (XD), and xylulokinase (XK) genes which are fused to promotors not inhibited by the presence of glucose.

Accordingly, one preferred embodiment of the invention provides a recombinant yeast strain containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and capable of fermenting xylose to ethanol. The recombinant yeast strain is preferably also capable of fermenting glucose to ethanol, and more preferred such yeast strains which can effectively ferment these two sugars simultaneously to ethanol are achieved where the XR, XD and XK genes are fused to promotors which are not inhibited by the presence of glucose and also do not require xylose for induction.

Another preferred embodiment of the invention provides a recombinant yeast strain containing genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase, wherein said genes are fused to non-glucose-inhibited promotors and wherein said yeast is capable of fermenting xylose to ethanol. The recombinant yeast strain is preferably also capable of fermenting glucose to ethanol.

Other preferred embodiments of the invention relate to reagents useful for the production of recombinant

yeasts of the invention. Thus, the present invention also provides a recombinant DNA molecule comprising genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase. As well, the invention provides a vector comprising genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase. In these reagents, the genes are preferably fused to promotors which are not inhibited by glucose and also do not require xylose for induction, so as to enable the expedient production of recombinant yeasts capable of simultaneously fermenting glucose and xylose to ethanol.

Another preferred embodiment of the present invention provides a method for obtaining a recombinant yeast capable of fermenting xylose to ethanol. This method includes the step of introducing DNA into a yeast so as to cause the yeast to have introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase. Preferably, these genes will be fused to non-glucose-inhibited promotors to enable simultaneous fermentation of glucose and xylose to ethanol. Advantageously, all three genes can be introduced simultaneously, for instance using reagents of the invention as discussed above.

Still other preferred embodiments of the invention provide methods for fermenting xylose or glucose to ethanol. The inventive methods include the step of fermenting a xylose-containing or glucose-containing medium with a recombinant yeast strain containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase. It is desirable that the three introduced genes be fused to non-glucose-inhibited promotors, and that the medium contain both glucose and xylose, so as to provide the concurrent fermentation of

xylose and glucose to ethanol.

Additional preferred embodiments, features and advantages of the invention will be apparent from the following description.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram of the enzymes associated with early stages of xylose metabolism in bacteria and yeasts.

Figure 2 shows the nucleotide sequence and deduced amino acid sequence of the yeast xylulokinase gene including its 5'- and 3'-flanking sequences. The initiation codon and stop codon are underlined. The possible control sequences in the 5' and 3' non-coding regions are indicated by arrows.

Figure 3 shows the genes cloned on and the restriction map of the plasmid pLSK15.

Figure 4 shows the genes cloned on and the restriction map of the plasmid pUCKm10.

Figure 5 shows the genes cloned on and the restriction map of the plasmid pLNH21.

Figure 6A shows an HPLC chromatogram of a fermentation broth obtained by fermenting xylose with recombinant yeast SC (pLNH21) (S. cerevisiae containing introduced XR, XD and XK genes) for (I) 2 days; and (II) 4 days.

Figure 6B shows an HPLC chromatogram of a fermentation broth obtained by fermenting xylose with recombinant yeast SC (pLNH13-32) (S. cerevisiae containing introduced XR and XD but not XK genes) for (I) 2 days; and (II) 6 days.

Figure 6C shows an HPLC chromatogram of a

fermentation broth obtained by fermenting xylose with an un-engineered S. cerevisiae yeast (containing no introduced XR, XD or XK genes) for (I) 2 days; and (II) 7 days, as further described in Example 6.

Figure 7 shows the genes cloned on and the restriction map of plasmid pLNH33.

Figure 8A shows an HPLC chromatogram of a fermentation broth obtained by fermenting a glucose- and xylose-containing medium (10% and 5%, respectively) with un-engineered yeast strain 1400 (containing no introduced XR, XD or XK genes) for (I) O days; and (II) 2 days, as further described in Example 8.

Figure 8B shows an HPLC chromatogram of a fermentation broth obtained by fermenting a glucose- and xylose-containing medium (10% and 5%, respectively) with recombinant yeast 1400 (pLNH33) (yeast 1400 containing introduced XR, XD and XK genes) for (I) 0 days; and (II) 2 days, as further described in Example 8.

Figure 9 is a schematic diagram outlining the construction of pBluescript II KS(-) containing the cloned XR, XD, and XK genes: four such plasmids were constructed: pKS(-)-KK-A*R-KD-1; pKS(-)-KK-A*R-KD-2; pKS(-)-KK-AR-KD-3; and pKS(-)-KK-AR-KD-4, as further described in Example 4.

Figure 10 shows direct amplification of the intact xylitol dehydrogenase gene and the promotorless XD from P. stipitis chromosomal DNA by the polymerase chain reaction (PCR) technique; from left, Lane 1: Molecular markers BamHI-EcoRI digested l DNA; Lane 2: Pichia xylitol dehydrogenase gene (intact); Lane 3: Pichia xylitol

dehydrogenase gene (promotorless); and Lane 4: Molecular markers, HaeIII digested ϕX DNA.

Figure 11 diagrams the strategies used for sequencing the yeast xylulokinase gene.

Figure 12 is a schematic diagram outlining the construction of the plasmid pLNH21.

Figure 13 shows an HPLC chromatogram of a fermentation broth obtained by fermenting a mixture of glucose (10%) and xylose (5%) with S. cerevesiae SC (pLNH13-32) (containing only the XR and XD genes) for (I) 0 days; (II) 2 days; and (III) 5 days.

Figure 14 shows an HPLC chromatogram of the fermentation broth obtained by fermenting a mixture of glucose (10%) and xylose (5%) with unengineered Pichia stipitis for (I) 0 days; (II) 3 days; and (III) 5 days.

DETAILED DESCRIPTION

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to certain embodiments thereof and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations, further modifications and applications of the principles of the invention as illustrated herein being contemplated as would normally occur to one skilled in the art to which the invention pertains.

The present invention provides recombinant yeasts, DNA molecules and vectors comprising XR, XD and XK genes. Such genes are well known to occur in a wide variety of microorganisms and, in fact, as discussed hereinabove, numerous XR, XD and XK genes have been identified and isolated. The particular source of these genes is not critical to the broad aspects of this invention; rather, any DNAs encoding proteins (enzymes) having xylose reductase activity (the ability to convert D-xylose to xylitol with NADPH or NADH as cofactor), xylitol dehydrogenase activity (the ability to convert xylitol to D-xylulose with NAD^+ as cofactor), or xylulokinase activity (the ability to convert D-xylulose to D-xylulose-5-phosphate) will be suitable. These genes may be obtained as naturally-occurring genes, or may be modified, for example, by the addition, substitution or deletion of bases to or of the naturally-occurring gene, so long as the encoded protein still has XR, XD or XK activity. Similarly, the genes or portions thereof may be synthetically produced by known techniques, again so long as the resulting DNA encodes a protein exhibiting the desired XR, XD or XK activity.

As examples, suitable sources of XR and XD genes include xylose-utilizing yeasts such as Candida shehatae, Pichia stipitis, Pachysolen tannophilus, suitable sources of XK genes include the above-noted xylose-utilizing yeasts, as well a xylose non-utilizing yeasts such as those from the genus Saccharomyces, e.g. S. cerevisiae, the genus Schizosaccharomyces, e.g. Schizosaccharomyces pombe, and bacteria such as Escherichia coli, Bacillus species, Streptomyces species, etc. Genes of interest can be recovered from these sources utilizing conventional methodologies. For example, hybridization, complementation or PCR techniques can be employed for this purpose.

The particular XR gene used in the applicants' studies herein was cloned from P. stipitis by Polymerase Chain Reaction (PCR) (Chen and Ho, 1993). The oligonucleotides required for the amplification of XR from the chromosomal DNA by PCR were synthesized according to the published sequence of the P. stipitis XR gene (Takama et al., 1991). The amplified XR was first cloned and stored into plasmid pUC19. The cloned XR was then fused to different promotors including the promotors of yeast TRP5 gene (Zalkin and Yanofsky, 1982) and yeast alcohol dehydrogenase I gene (ADC1) (Ammerer, 1983; Bennetzen and Hall, 1982).

The XD gene used in the applicants' studies was also cloned from P. stipitis by PCR. The oligonucleotides required for the amplification of XD from the Pichia chromosomal DNA were synthesized according to the published sequence of the Pichia XD gene (Köetter et al., 1990). The amplified XD was also first cloned and stored in pUC19. The gene was then subsequently fused to

glycolytic promotors of yeast pyruvate kinase gene (PYK) (Burke et al., 1983) and yeast glyceraldehyde 3 phosphodehydrogenase gene (GPD) (Holland and Holland, 1979).

The applicants have cloned three different XK genes, those from S. cerevisiae (Ho and Chang, 1989), P. tannophilus (Stevis et al., 1987) and E. coli and have found that all three genes can be effectively expressed in S. cerevisiae after fusion to a highly efficient yeast promoter. The cloned S. cerevisiae xylulokinase gene was used in the illustrative work set forth herein. To assist in properly fusing the yeast XK gene to a suitable promoter, the complete nucleotide sequence of the S. cerevisiae XK gene including its 5' and 3' non-coding sequence has been analyzed and is shown in Figure 2.

A wide variety of promotors will be suitable for use in the invention. Broadly speaking, yeast-compatible promotors capable of controlling transcription of the XR, XD or XK genes will be used. Such promotors are available from numerous known sources, including yeasts, bacteria, and other cell sources. Preferably, the promotors used in the invention will be efficient, non-glucose-inhibited promotors, which do not require xylose for induction. In this regard, an "efficient" promotor as used herein refers to a promotor which provides a high level of transcription of the fused gene. Promotors having these characteristics are also widely available, and their use in the present invention, given the teachings herein, will be within the purview of the ordinarily skilled artisan, as will be the fusion of the promotors to the XR, XD and XK genes, the cloning of the promotor/gene fusion products into appropriate vectors and the use of the vectors to transform yeast. All of these manipulations can be

performed using conventional genetic engineering techniques well known to the art and literature.

More particularly describing the applicant's illustrative work herein, the yeast xylulokinase gene, XK, has been fused to promotors from yeast alcohol dehydrogenase gene (ADC1), yeast pyruvate kinase gene (PYK), yeast TRP5-gene, etc. XK fused to the TRP-5 promoter was used to construct pLNH21 (Figure 5) and XK fused to the PYK promotor was used to construct pLNH33 (Figure 7).

The fusion of XR, XD, and XK to intact promotors from ADC1, PYK, GPD, etc., was carried out by cloning both the fragment containing the specific promoter and the structural gene of XR, XD, or XK on one of the Bluescript KS plasmids (Stratagene, La Jolla, CA), followed by the removal of the extra unwanted nucleotides by site-specific mutagenesis (Kunkel et al., 1987). The invention thus also provides several pBluescript II KS(-) (hereinafter pKS(-)) derivatives containing the cloned XD (fused to the pyruvate dehydrogenase promoter), XR (fused to the ADC1 promoter), and XK (fused to the pyruvate kinase promoter). These recombinant plasmids are designated as pKS(-) KD-AR (or A*R) -KK. Four such plasmids were constructed as outlined in Figure 9. These plasmids have similar but not identical structures. The XR, XD, and XK (or KD-AR (or A*R) -KK) cloned on these plasmids can be separated from the parent pKS(-) plasmid by a single XhoI restriction digestion.

The XR, XD, and XK genes fused to the proper promotors were then cloned on pLSK15 (Figure 3) or pUCKm10 (Figure 4). pLSK15, a derivative of pLX10-14 (Stevis and Ho, 1985), is a low copy number plasmid with a copy number

of approximately 10 in yeast (S. cerevisiae). It contains the yeast 2μ replicon which enables the plasmid to be replicated autonomously in S. cerevisiae and closely related species. pLSK15 also contains the geneticin (kanamycin) resistance gene (Km^R) and ampicillin resistance gene (Ap^R and also amp^r) which serve as selection markers in S. cerevisiae and other yeasts. pLSK15 also contains the XK gene fused to the yeast TRP-5 promoter. Thus, XR and XD genes fused to proper 5' noncoding sequences containing suitable promotors were inserted into pLSK15 to demonstrate the effect of the resulting plasmids on yeast xylose fermentation. To compare the effect of the presence of different genes on yeast xylose fermentation, a plasmid containing only XR and XD was also used to transform S. cerevisiae and the resulting yeast used in comparative fermentations. Results of the fermentation of xylose by un-engineered S. cerevisiae, yeast containing the cloned XR, XD, and XK (SC(pLNH21)), and yeast containing the cloned XR and XD but not XK (SC(pLNH13-32)) genes are shown in Figure 6A, 6B, and 6C.

pUCKm10 (Figure 4) is a high copy-number plasmid (i.e. plasmid with a copy number of about 50 or more) with a copy number close to 100 in S. cerevisiae. pUCKm10 is a pUC9 derivative containing the identical 2μ replicon, and the Km^R, and Ap^R genes present in pLSK15. These specific DNA fragments serve as the replicon and selection markers that enable the plasmid to be replicated autonomously in S. cerevisiae (and in related yeasts) and also enable the yeast transformants containing the plasmid to be distinguished from the untransformed host cells.

The applicants have constructed pUCKm10 based recombinant plasmids that contain the same XR, XD, and XK

fused to 5' proper noncoding sequences containing suitable promotors. These vectors are designed to be useful to transform all S. cerevisiae strains and strains of related species. No special mutants are required to act as the recipient strains. Thus plasmids such as pLNH33 (Figure 7), as well as pLNH21 (Figure 5), can be used to transform industrial S. cerevisiae and other strains.

Yeast transformation with derivatives of either pLSK15 or pUCKm10 was carried out by electroporation generally using the the procedure described by Becker and Guarente (1991). Authentic yeast transformants containing derivatives of either pLSK15 or pUCKm10 were isolated as further described below. Km^R present in the plasmids served as the primary selection marker which renders any host cells obtaining one of these plasmids resistant to a much higher concentration of geneticin present in the medium. However, some yeast cells can be induced to become resistant to the same level of geneticin of the transformants containing the plasmid. Thus, not every geneticin resistant colony is a true transformant. It has been reported that Ap^R can be expressed in S. cerevisiae but the latter is resistant to ampicillin without the presence of Ap^R. Thus, Ap^R cannot serve as a selection marker for yeast plasmid-mediated transformation. Nevertheless, yeasts that contain the highly expressed Ap^R will produce sufficient penicillinase and make it possible to identify colonies containing such yeasts on special solid plates by the penicillinase test (Chevallier and Aigle, 1979). The latter test has provided a technique to identify the true transformants of S. cerevisiae and other yeasts from the geneticin resistant colonies.

Yeast xylose (or xylose and glucose) fermentation was

carried out using the inventive recombinant yeasts under anaerobic conditions as described in Examples 6 through 9. The consumption of sugars (xylose and glucose) and the formation of ethanol and other products such as xylitol were followed during fermentation by taking samples and analyzing them by HPLC as further described in Example 6.

For example, pLNH21 (Figure 5) was used to transform S. cerevisiae. The resulting transformant containing pLNH21 is designated SC(pLNH21, and can ferment 5% xylose nearly totally to ethanol in two to four days as demonstrated in Figure 6A.

As an additional example, pLNH33 (Figure 7) was used to transform yeast strain 1400 which is closely related to S. cerevisiae and has high tolerance to alcohol and temperature (D'Amore et al., 1989; D'Amore, 1990). The resultant genetically engineered yeast, designated 1400(pLNH33), can ferment 10% glucose and 5% xylose totally to ethanol in two to four days, without requiring high cell densities, as shown in Figures 8A and 8B.

pLNH33 is a more effective plasmid than pLNH21 for xylose fermentation because it is a higher copy-number plasmid. Furthermore, the XK in pLNH33 is fused to a more efficient promoter than the XK in pLNH21. S. cerevisiae has also been transformed with pLNH33, designated SC(pLNH33). Although SC(pLNH33) is much more effective in fermenting xylose or mixtures of xylose and glucose than SC(pLNH21), 1400(pLNH33) was found to be more effective in fermenting mixtures of glucose and xylose than SC(pLNH33). Thus, individual strains also affect the efficiency of fermentation. Similar to S. cerevisiae, the unengineered strain 1400 cannot use or ferment xylose (alone or in a mixture of glucose and xylose) as shown in

Figure 8B.

Generally, the results of these fermentive tests demonstrate that it is necessary that the yeast contain three introduced genes, XR, XD, and XK which have been properly fused to suitable promotors (preferably efficient glycolytic or other promotors that are not subject to glucose inhibition, and do not require xylose for induction) and to coordinately express these genes to make the yeast capable of fermenting xylose to ethanol only, and not to other by-products such as xylitol.

The results further demonstrate the importance of cloning a xylulokinase gene (XK) in addition to XR and XD in order to make yeasts ferment xylose effectively, particularly to ferment both glucose and xylose simultaneously when they are present in the same medium, such as in the hydrolyzates of cellulosic biomass. Similar to XR and XD, the cloned XK is preferably fused to a suitable efficient glycolytic or other promoter that is not subject to glucose inhibition, and which further does not require xylose for induction.

Also, the applicants found that yeast containing just the cloned XR an XD can only ferment glucose but not xylose to ethanol when both these sugars are present in the culture medium together (see Figure 13). Moreover, the applicants' results demonstrate that it is necessary for any yeast, including those xylose fermenting yeasts such as P. stipitis and C. shihatae to contain XR, XD and XK, fused to promotors that are not inhibited by the presence of glucose and also not requiring the use of xylose for induction in order to be able to ferment both glucose and xylose to ethanol when both these sugars are present together in the culture medium. Figure 13

demonstrates that S. cerevisiae and related species containing only the cloned XR and XD genes, fused to proper promotors, can only ferment glucose but not xylose to ethanol when both these sugars are present in the culture medium. Similarly, Figure 14 demonstrates that unengineered P. stipitis containing its original XR, XD, and XK can ferment xylose when the latter sugar is the sole carbon source of the medium (results not shown) but it cannot ferment xylose when both glucose and xylose are the carbon sources present in the same medium.

It will be understood that for those yeasts that contain low levels of xylulokinase activity, introducing the XK gene serves two purposes. One is to improve the level of the enzyme activity. High levels of XK activity are important for more advantageous yeast fermentation of xylose to ethanol as opposed to xylitol. The other is to place the gene under the control of an efficient promoter that will not be inhibited by the presence of glucose. It is well known that natural wild-type microorganisms including yeasts cannot use other sugars for growth and fermentation if glucose is present in the cultural medium. Glucose will inhibit the synthesis of the enzymes required for metabolizing other sugar molecules (the so called "glucose" effect). Thus promotors from genes for the synthesis of sugar molecule metabolizing enzymes excluding glucose will not be preferred since these will not provide simultaneous fermentation of the two abundant sugars. In addition, it was found in the applicants' work that cell growth is also a prerequisit for induction. Thus, promotors requiring xylose for induction are not preferred for the expression of XR, XD or XK.

For the purpose of promoting a further understanding of the present invention and its advantages, the following

Examples are provided. It will be understood that these Examples are illustrative, and not limiting, in nature.

EXAMPLE 1
Synthesizing the XR and XD genes by PCR.

The synthesis of the intact or promotorless XR by PCR has been previously described (Chen and Ho, 1993). For the synthesis of XD by PCR, three oligonucleotides according to the nucleotide sequence of XD (Köetter et al., 1990) were synthesized and are listed below:

Oligonucleotide I: pTCTAGACCACCCTAAGTCG
Oligonucleotide I1: pCACACAATTAAAATGA
Oligonucleotide III: pGGATCCACTATAGTCGAAG

Oligonucleotides I and II were used to synthesize the intact XD gene and oligonucleotides II and III were used to synthesize the promotorless XD as shown in Figure 10. The intact XD and the promotorless XD were first cloned in pKS(-) plasmid. The intact XR was then subcloned on pUCKm10 (Figure 4) and the resulting plasmid pUCKm10-XD, was used to transform S. cerevisiae by electroporation as described in Example 5. The yeast transformants were used to assay the xylitol dehydrogenase activity to demonstrate that the cloned gene is intact and can be expressed in S. cerevisiae.

EXAMPLE 2
Fusion of the promotorless XD gene to the yeast pyruvate kinase gene promotor

Fusion of the XD gene to P_{PK} was chosen to illustrate the precise fusion of xylose metabolizing genes to intact promotors by site-directed mutagenesis. These promotors are either glycolytic promotors or promotors

that will not be inhibited by the presence of glucose in the culture medium and also will not require the presence of xylose for induction.

The promoter fragment of yeast pyruvate kinase from -910 to +23 (Burke et al., 1983) was synthesized by PCR as described in Example 1 for the synthesis of the XD gene. Both the P_{PK} fragment and the promotorless XD were subcloned on pKS(-) plasmid and the undesired nucleotides between the P_{PK} and the intact XD structural gene were removed by site-specific mutagenesis according to the procedure of Kunkel (Kunkel, 1987). The resulting fused gene contains -910 to -1 promoter fragments from the pyruvate kinase gene and +1 to +1963 nucleotides from the Pichia XD gene. The resulting pKS(-) plasmid containing P_{PK}-XD (or KD) is designated pKS(-)-KD or pKD2.

EXAMPLE 3
Analysis of the nucleotide sequence of yeast xylulokinase gene

The cloning of a 7.0 kb yeast (S. cerevisiae) DNA fragment that contains the yeast xylulokinase gene has been previously reported (Ho and Chang, 1989). By subcloning, the XK gene has been located on a 2.4 kb fragment. The nucleotide sequence of the 2.4 kb fragment has been analyzed. The 5' non-coding region contains 345 nucleotides, the translated region contains 2118 nucleotides, and the xylulokinase encoded by XK has 591 amino acids as shown in Figure 2. The strategy used for sequencing the XK gene is shown in Figure 11.

EXAMPLE 4
Construction of intact ADC1 promoter

Plasmid pMA56 (Ammerer, 1983) contains the yeast

alcohol dehydrogenase I promoter (P_{ADC1}). The applicants have used this promoter to modify some of the genes in their work. For example, P_{ADC1} has been fused to XR, and the resulting gene has been designated P_{ADC1}-XR or AR. Nevertheless, this P_{ADC1} is not intact and does not contain the -1 to -14 nucleotides of the intact ADC1 promoter (Bennetzen and Hall, 1982). The -1 to -14 region of a gene is usually very significant for controlling protein synthesis. Any gene fused to such a promoter has to rely on its original genetic signal for controlling the synthesis of its protein product.

In order to better control the expression of the gene fused to the ADC1 promoter, the applicants employed site-specific mutagenesis to add the missing nucleotides (-1 to -14) to the ADC1 promoter cloned on pMA56. The new intact ADC1 promoter is designated P^{*}_{ADC1}. This promoter has been used to modify XR and the resulting gene is designated as P^{*}_{ADC1}-XR or $A^{*}R$.

EXAMPLE 5
Construction of plasmid pLNH21 (also designated as pLSK15-KD-AR) and transformation of S. cerevisiae and 1400 with pLNH21

The construction of pLNH21 is outlined in Figure 12. pLNH21 was used to transform S. cerevisiae and strain 1400 by electroporation under the following conditions. Fifty ml yeast cells, grown to early log phase (Klett Unit (KU) 130), were centrifuged to remove the medium, washed twice with cold water, once with cold 1 M sorbitol, and resuspended in 200 µl 1 M sorbitol. Sixty µl of the cells were transferred into a 4 ml presterilized plastic tube (with cap) and to which 0.1 µg to 1 µg plasmid DNA was added. Fifty µl of the resulting cells and

plasmid mixture were pipetted into a precooled gene pulser cuvette with a 0.2 cm electrode gap and the content in the cuvette was subjected to pulse by the gene pulser with a pulse controller (BioRad) at 2.0 KV, 25 µF, 200 ohms.

Immediately, .50 ml YEPD (1% yeast extract, 2% peptone, and 2% glucose) was added to the cuvette. The content of the cuvette was transferred to a new 4 ml sterilized plastic tube and incubated at 30°C for 1 hr. 100 µl of the cells were plated on agar plates containing YEPD and 50 µg/ml G418 (geneticin). Fast growing colonies were selected and replicated on another plate containing the same medium. The selected colonies were subjected to the ampicillin test (Chevallier and Aigle, 1979) until a positive one was identified. The above-described electroporation procedure is based on that reported by Becker and Guarente (1971). Our method for the selection of G418 resistant transformants is very effective and most of the selected colonies that were replicated on plates containing YEPD plus 50 µg/ml G418 were positive for the penicillinase test.

Transformation of strain 1400 with pLNH21 or other plasmids was carried out using a similar procedure to that described above, except that the cells were grown to 140-190 KU rather than 130 KU and the YEPD plates for the initial selection of transformants after electroporation contained 40 µg/ml geneticin G418 rather than 50. Transformation of strain 1400 by the above described procedures was not as effective as transformation of S. cerevisiae.

EXAMPLE 6
Fermentation of xylose with engineered SC(pLNH21), SC(pLNH13-32), and un-engineered parent S. cerevisiae

These three yeasts were cultured in rich medium YEPD aerobically under identical conditions (SC(pLNH13-32) was constructed by transforming S. cerevisiae with a plasmid, designated pLNH13-32, which contains only the XR and XD gene/promotor combinations). These yeast cells were then used to ferment 5% xylose in YEP (1% yeast extract, 2% peptone) medium anaerobically also under identical conditions. The consumption of xylose and the formation of ethanol and xylitol were followed during fermentation by taking samples at proper intervals and analyzing them by HPLC under the following conditions.

The samples containing the fermentation broth (0.6 ml to 1.0 ml) removed from the cultures were kept in 1.5 ml Eppendorf tubes. The cells and other residues were first removed by centrifugation. The supernatant was further filtered by using sterile aerodisc (Gelman Sciences), 0.2 or 0.45 mm, syringe filters. The resulting filtrate from each sample was analyzed for its ethanol, glucose, xylose, and xylitol contents by high performance liquid chromatography (HPLC), using a Hitachi system according to the following conditions.

°Column: Aminex HPX-87C, 300 X 7.8 mm
°Mobile phase: water
°Flow rate: 0.8 ml/min.
°Detection: Hitachi L-3350 RI detector
°Temperature: 80°C
°Injection volume: 20 µl

The results, shown in Figures 6A, 6B, and 6C (ethanol peaks in these and other Figures are actually 2 1/2 times smaller than they should be due to the sensitivity of the instrument), demonstrate that only the engineered yeast SC(pLNH21) containing the cloned XR, XD, and XK can

ferment high concentrations of xylose (5%) to ethanol, not the un-engineered parent S. cerevisiae, and also not the engineered SC(pLNH13-32) which only contains the cloned XD and XR, not XK. SC(pLNH13-32) ferments xylose mostly to xylitol.

EXAMPLE 8
Effective fermentation of high concentrations of both glucose and xylose by 1400(pLNH33) to ethanol

A mixture of glucose and xylose (approximately 10% glucose and 5% xylose) were fermented by strain 1400 and 1400(pLNH33) under identical conditions. These yeasts were kept on agar plates containing the proper media and were inoculated directly from the agar plates into 50 ml of YEPD medium (1% Yeast extract, 2% peptone, and 2% glucose) in a 250 ml Erlenmeyer flask equipped with a side-arm which allows direct monitoring of the growth of the yeast cultures by the Klett colorimeter. The cultures were incubated in a shaker at 30°C and 200 rpm aerobically.

When the cell density reached mid-log phase (400 Klett units), 12.5 ml (40%) glucose and 6.25 ml (40%) xylose were added to each flask. After thorough mixing, 1 ml of the culture mixture was removed from the flask to serve as the zero sample. The flask was then sealed with Saran wrap to allow fermentation to be carried out anaerobically. One ml samples of the fermentation broth (with some cells) were removed at proper intervals (every 24 hr.) to serve as samples for measuring the sugar and ethanol contents of the broth during fermentation. The ethanol, glucose, xylose, and xylitol contents of the samples were analyzed by HPLC as described in Example 6. The results, shown in Figures 8A and 8B, demonstrate that the genetically engineered yeast 1400(pLNH33) can ferment 10% glucose and 5% xylose to ethanol simultaneously in two

to four days without requiring high cell density. On the other hand, the parent strain 1400 can only convert glucose to ethanol but not xylose. The fermentation was carried out under normal conditions, without requiring special medium, special pH, and also without requiring growth of yeast to high cell density. Thus the genetically engineered 1400(pLNH33) containing the XR, XD, and XK, all fused to glycolytic promotors and cloned on a high copy-number plasmid pUCKm10, can ferment high concentrations of both glucose and xylose simultaneously to ethanol in two to four days with very little xylitol produced as a by-product.

EXAMPLE 9
Attempted Fermentation of xylose/glucose with engineered SC(pLNH13-32)

The fermentation procedure of Example 8 was repeated except using S. cerevisiae SC (pLNH13-32) (containing only the XR and XD genes) as the fermentive organism. The results, shown in Figure 13, demonstrate that such a genetic unengineered yeast containing only the XR and XD genes can ferment glucose but not xylose when both of these sugars are present in the fermented medium.

EXAMPLE 10
Attempted Fermentation of xylose/glucose with unengineered Pichia stipitis

The fermentation procedure of Example 8 was repeated, except using unengineered Pichia stipitis as the fermentive organsim. Samples of the fermentation broth were analyzed by HPLC after fermentation for (I) 0 day; (II) 3 days; and (III) 5 days. The results, shown in Figure 14, demonstrate that P. stipitis can only ferment glucose, but not xylose when both of these sugars are

present in the same medium.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected.

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(2) Information for Seq ID No:1:
(i) Sequence Characteristics
(A) Length: 2467 base pairs
(B) Type: Nucleotide Amino Acid
(C) Strandedness: Double
(D) Topology: Linear
(ii) Molecule Type: Genomic DNA

(xi) Sequence Description: SEQ ID NO:1:

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AACTGTCACT TTTCAATTCG GCCAATGCAA TCTCAGGCGG ACGAATAAGG     300
GGGCCCTCTC GAGAAAAACA AAAGGAGGAT GAGATTAGTA CTTTA ATG TTG  351
                                                  Met Leu
                                                    1
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Met Ser Leu Asp Ser Tyr Tyr Leu Gly Phe Asp Leu Ser Thr
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CAA CAA CTG AAA TGT CTC GCC ATT AAC CAG GAC CTA AAA ATT      477
Gln Gln Leu Lys Cys Leu Ala Ile Asn Gln Asp Leu Lys Ile
                    35                  40

GTC CAT TCA GAA ACA GTG GAA TTT GAA AAG GAT CTT CCG CAT      519
Val His Ser Glu Thr Val Glu Phe Glu Lys Asp Leu Pro His
45                  50                  55

TAT CAC ACA AAG AAG GGT GTC TAT ATA CAC GGC GAC ACT ATC      561
Tyr His Thr Lys Lys Gly Val Tyr Ile His Gly Asp Thr Ile
     60                  65                  70

GAA TGT CCC GTA GCC ATG TGG TTA GGG GCT CTA GAT CTG GTT      603
Glu Cys Pro Val Ala Met Trp Leu Gly Ala Leu Asp Leu Val
          75                  80                  85

CTC TCG AAA TAT CGC GAG GCT AAA TTT CCA TTG AAC AAA GTT      645
Leu Ser Lys Tyr Arg Glu Ala Lys Phe Pro Leu Asn Lys Val
               90                  95                  100

ATG GCC GTC TCA GGG TCC TGC CAG CAG CAC GGG TCT GTC TAC      687
Met Ala Val Ser Gly Ser Cys Gln Gln His Gly Ser Val Tyr
                    105                 110

TGG TCC TCC CAA GCC GAA TCT CTG TTA GAG CAA TTG AAT AAG      729
Trp Ser Ser Gln Ala Glu Ser Leu Leu Glu Gln Leu Asn Lys
115                 120                 125

AAA CCG GAA AAA GAT TTA TTG CAC TAC GTG AGC TCT GTA GCA      771
Lys Pro Glu Lys Asp Leu Leu His Tyr Val Ser Ser Val Ala
     130                 135                 140

TTT GCA AGG CAA ACC GCC CCC AAT TGG CAA GAC CAC AGT ACT      813
Phe Ala Arg Gln Thr Ala Pro Asn Trp Gln Asp His Ser Thr
          145                 150                 155

GCA AAG CAA TGT CAA GAG TTT GAA GAG TGC ATA GGT GGG CCT      855
Ala Lys Gln Cys Gln Glu Phe Glu Glu Cys Ile Gly Gly Pro
               160                 165                 170

GAA AAA ATG GCT CAA TTA ACA GGG TCC AGA GCC CAT TTT AGA      897
Glu Lys Met Ala Gln Leu Thr Gly Ser Arg Ala His Phe Arg
                    175                 180
```

```
TTT ACT GGT CCT CAA ATT CTG AAA ATT GCA CAA TTA GAA CCA      939
Phe Thr Gly Pro Gln Ile Leu Lys Ile Ala Gln Leu Glu Pro
185                 190                 200

GAA GCT TAC GAA AAA ACA AAG ACC ATT TCT TTA GTG TCT AAT      981
Glu Ala Tyr Glu Lys Thr Lys Thr Ile Ser Leu Val Ser Asn
    205                 210                 215

TTT TTG ACT TCT ATC TTA GTG GGC CAT CTT GTT GAA TTA GAG      1023
Phe Leu Thr Ser Ile Leu Val Gly His Leu Val Glu Leu Glu
        220                 225                 230

GAG GCA GAT GCC TGT GGT ATG AAC CTT TAT GAT ATA CGT GAA      1065
Glu Ala Asp Ala Cys Gly Met Asn Leu Tyr Asp Ile Arg Glu
            235                 240                 245

AGA AAA TTC ATG TAT GAG CTA CTA CAT CTA ATT GAT AGT TCT      1107
Arg Lys Phe Met Tyr Glu Leu Leu His Leu Ile Asp Ser Ser
                250                 255

TCT AAG GAT AAA ACT ATC AGA CAA AAA TTA ATG AGA GCA CCC      1149
Ser Lys Asp Lys Thr Ile Arg Gln Lys Leu Met Arg Ala Pro
260                 265                 270

ATG AAA AAT TTG ATA GCG GGT ACCA TCT GTA AA TAT TTT ATT      1191
Met Lys Asn Leu Ile Ala Gly Thr Ile Cys Lys Tyr Phe Ile
    275                 280                 285

GAG AAG TAC GGT TTC AAT ACA AAC TGC AAG GTC TCT CCC ATG      1233
Glu Lys Tyr Gly Phe Asn Thr Asn Cys Lys Val Ser Pro Met
        290                 300                 305

ACT GGG GAT ATT TTA GCC ACT ATA TGT TCT TTA CCC CTG CGG      1275
Thr Gly Asp Asn Leu Ala Thr Ile Cys Ser Leu Pro Leu Arg
            310                 320                 325

AAG AAT GAC GTT CTC GTT TCC CTA GGA ACA AGT ACT ACA GTT      1317
Lys Asn Asp Val Leu Val Ser Leu Gly Thr Ser Thr Thr Val
                330                 335

CTT CTG GTC ACC GAT AAG TAT CAC CCC TCT CCG AAC TAT CAT      1359
Leu Leu Val Thr Asp Lys Tyr His Pro Ser Pro Asn Tyr His
340                 345                 350

CTT TTC ATT CAT CCA ACT CTG CCA AAC CAT TAT ATG GGT ATG      1401
Leu Phe Ile His Pro Thr Leu Pro Asn His Tyr Met Gly Met
    355                 360                 365

ATT TGT TAT TGT AAT GGT TCT TTG GCA AGG GAG AGG ATA AGA      1443
Ile Cys Tyr Cys Asn Gly Ser Leu Ala Arg Glu Arg Ile Arg
        370                 375                 380
```

GAC GAG TTA AAC AAA GAA CGG GAA AAT AAT TAT GAG AAG ACT 1485
Asp Glu Leu Asn Lys Glu Arg Glu Asn Asn Tyr Glu Lys Thr
385 390 400

AAC GAT TGG ACT CTT TTT AAT CAA GCT GTG CTA GAT GAC TCA 1527
Asn Asp Trp Thr Leu Phe Asn Gln Ala Val Leu Asp Asp Ser
405 410

GAA AGT AGT GAA AAT GAA TTA GGT GTA TAT TTT CCT CTG GGG 1569
Glu Ser Ser Glu Asn Glu Leu Gly Val Tyr Phe Pro Leu Gly
415 420 425

GAG ATC GTT CCT AGC GTA AAA GCC ATA AAC AAA AGG GTT ATC 1611
Glu Ile Val Pro Ser Val Lys Ala Ile Asn Lys Arg Val Ile
430 435 440

TTC AAT CCA AAA ACG GGT ATG ATT GAA AGA GAG GTG GCC AAG 1653
Phe Asn Pro Lys Thr Gly Met Ile Glu Arg Glu Val Ala Lys
445 450 455

TTC AAA GAC AAG AGG CAC GAT GCC AAA AAT ATT GTA GAA TCA 1695
Phe Lys Asp Lys Arg His Asp Ala Lys Asn Ile Val Glu Ser
460 465 470

CAG GCT TTA AGT TGC AGG GTA AGA ATA TCT CCC CTG CTT TCG 1737
Gln Ala Leu Ser Cys Arg Val Arg Ile Ser Pro Leu Leu Ser
475 480

GAT TCA AAC GCA AGC TCA CAA CAG AGA CTG AAC GAA GAT ACA 1779
Asp Ser Asn Ala Ser Ser Gln Gln Arg Leu Asn Glu Asp Thr
485 490 495

ATC GTG AAG TTT GAT TAC GAT GAA TCT CCG CTG CGG GAC TAC 1821
Ile Val Lys Phe Asp Tyr Asp Glu Ser Pro Leu Arg Asp Tyr
500 505 510

CTA AAT AAA AGG CCA GAA AGG ACT TTT TTT GTA GGT GGG GCT 1863
Leu Asn Lys Arg Pro Glu Arg Thr Phe Phe Val Gly Gly Ala
515 520 525

TCT AAA AAC GAT GCT ATT GTG AAG AAG TTT GCT CAA GTC ATT 1905
Ser Lys Asn Asp Ala Ile Val Lys Lys Phe Ala Gln Val Ile
530 535 540

GGT GCT ACA AAG GGT AAT TTT AGG CTA GAA ACA CCA AAC TCA 1947
Gly Ala Thr Lys Gly Asn Phe Arg Leu Glu Thr Pro Asn Ser
545 550

TGT GCC CTT GGT GGT TGT TAT AAG GCC ATG TGG TCA TTG TTA 1989
Cys Ala Leu Gly Gly Cys Tyr Lys Ala Met Trp Ser Leu Leu
555 560 565

```
TAT GAC TCT AAT AAA ATT GCA GTT CCT TTT GAT AAA TTT CTG      2031
Tyr Asp Ser Asn Lys Ile Ala Val Pro Phe Asp Lys Phe Leu
    570                 575                 580

AAT GAC AAT TTT CCA TGG CAT GTA ATG GAA AGC ATA TCC GAT      2073
Asn Asp Asn Phe Pro Trp His Val Met Glu Ser Ile Ser Asp
        585                 590                 595

GTG GAT AAT GAA AAT TGG ATC GCT ATA ATT CCA AGA TTG TCC      2115
Val Asp Asn Glu Asn Trp Ile Ala Ile Ile Pro Arg Leu Ser
            600                 605                 610

CCT TAAGCGAACT GGAAAAGACT CTCATCTAAA ATATGTTTGA ATAATTTATC   2168
Pro

ATGCCCTGAC AAGTACACAC AAACACAGAC ACATAATATA CATACATATA       2218
TATATATCAC CGTTATTATG CGTGCACATG ACAATGCCCT TGTATGTTTC       2268
GTATACTGTA GCAAGTAGTC ATCATTTTGT TCCCCGTTCG GAAAATGACA       2318
AAAAGTAAAA TCAATAAATG AAGAGTAAAA AACAATTTAT GAAAGGGTGA       2368
GCGACCAGCA ACGAGAGAGA CAAATCAAAT TAGCGCTTTC CAGTGAGAAT       2418
ATAAGAGAGC ATTGAAAGAG CTAGGTTATT GTTAAATCAT CTCGAGCTC        2467
```

What is claimed is:

1. A recombinant yeast containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and effective for fermenting xylose to ethanol.

2. The recombinant yeast of claim 1 wherein the yeast is also effective for fermenting glucose to ethanol.

3. The recombinant yeast of claim 2 wherein the yeast is of the genus Saccharomyces.

4. The recombinant yeast of claim 3 wherein said genes are fused to non-glucose-inhibited promotors and the yeast is effective for simultaneously fermenting glucose and xylose to ethanol.

5. A recombinant DNA molecule comprising genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase.

6. The recombinant DNA molecule of claim 5 wherein said genes are fused to non-glucose-inhibited promotors.

7. A vector effective for transforming yeast and comprising genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase.

8. The vector of claim 7 wherein said genes are fused to non-glucose-inhibited promotors.

9. A method for obtaining a recombinant yeast effective for fermenting xylose to ethanol, comprising introducing DNA into a yeast so as to cause the yeast to

have introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase.

10. The method of claim 9 wherein said introduced DNA comprises genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase.

11. The method of claim 9 wherein said yeast is of the genus Saccharomyces.

12. A method for fermenting xylose to ethanol, comprising fermenting a xylose-containing medium with a recombinant yeast containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and effective for fermenting xylose to ethanol.

13. The method of claim 10 wherein the medium also contains glucose and the yeast is also effective for fermenting said glucose to ethanol.

14. The method of claim 13 wherein the yeast is of the genus Saccharomyces.

15. The method of claim 14 wherein said genes are fused to non-glucose-inhibited promotors and the yeast is effective for simultaneously fermenting glucose and xylose to ethanol.

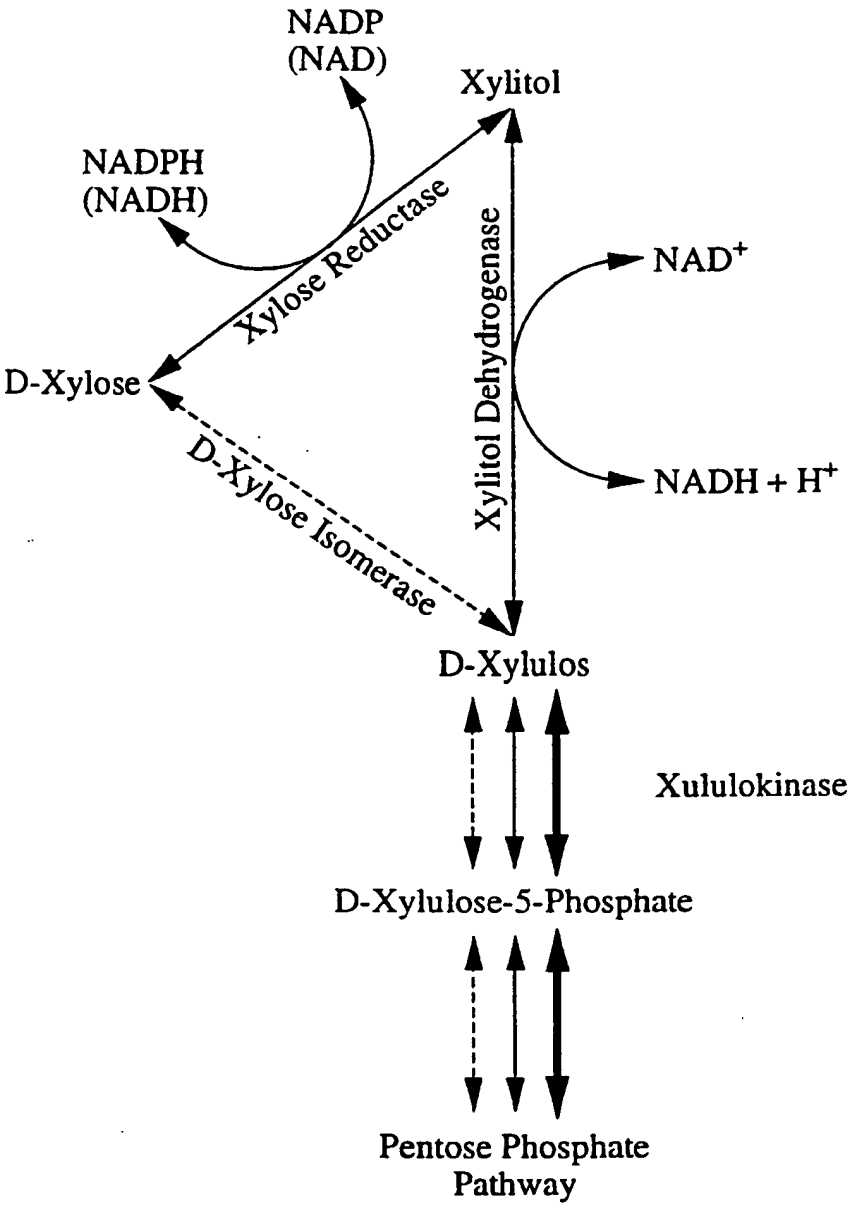
16. A method for fermenting glucose to ethanol, comprising fermenting a glucose-containing medium with a recombinant yeast containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and effective for fermenting xylose and glucose to ethanol.

17. The method of claim 16 wherein said medium also contains xylose.

18. The method of claim 17 wherein said yeast is of the genus Saccharomyces.

19. A recombinant yeast containing genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase, wherein said genes are fused to non-glucose-inhibited promotors and wherein said yeast is effective for fermenting xylose to ethanol.

20. The recombinant yeast of claim 19 wherein said yeast is also effective for fermenting glucose to ethanol.



The xylose metabolic pathways in microorganisms.

——— Xylose non-utilizing yeasts (*Saccharomyces cerevisiae, Schizosaccharomyces pombe,* etc.)

——— Xylose utilizing yeasts (*Candida shehatae, Pichia stipitis, Pachysolen tannophilus,* etc.)

--------- Bacteria (*E. coli, Bacillus* species, *Streptomyces* species, etc.)

Figure 1.

```
1    GGATCCAAGACCATTATTCCATCAGAATGGAAAAAAGTTTAAAAGATCACGGAGATTTTG

61   TTCTTCTGAGCTTCTGCTGTCCTTGAAAACAAATTATTCCGCTGGCCGCCCCAAACAAAA

121  ACAACCCCGATTTAATAACATTGTCACAGTATTAGAAATTTTCTTTTTACAAATTACCAT
                                  →

181  TTCCAGCTTACTACTTCCTATAATCCTCAATCTTCAGCAAGCGACGCAGGGAATAGCCGC
                                  →

241  TGAGGTGCATAACTGTCACTTTTCAATTCGGCCAATGCAATCTCAGGCGGACGAATAAGG

301  GGGCCCTCTCGAGAAAAACAAAAGGAGGATGAGATTAGTACTTTAATGTTGTGTTCAGTA
                                                 M  L  C  S  V

361  ATTCAGAGACAGACAAGAGAGGTTTCCAACACAATGTCTTTAGACTCATACTATCTTGGG
      I  Q  R  Q  T  R  E  V  S  N  T  M  S  L  D  S  Y  Y  L  G

421  TTTGATCTTTCGACCCAACAACTGAAATGTCTCGCCATTAACCAGGACCTAAAAATTGTC
      F  D  L  S  T  Q  Q  L  K  C  L  A  I  N  Q  D  L  K  I  V

481  CATTCAGAAACAGTGGAATTTGAAAAGGATCTTCCGCATTATCACACAAAGAAGGGTGTC
      H  S  E  T  V  E  F  E  K  D  L  P  H  Y  H  T  K  K  G  V

541  TATATACACGGCGACACTATCGAATGTCCCGTAGCCATGTGGTTAGGGGCTCTAGATCTG
      Y  I  H  G  D  T  I  E  C  P  V  A  M  W  L  G  A  L  D  L

601  GTTCTCTCGAAATATCGCGAGGCTAAATTTCCATTGAACAAAGTTATGGCCGTCTCAGGG
      V  L  S  K  Y  R  E  A  K  F  P  L  N  K  V  M  A  V  S  G

661  TCCTGCCAGCAGCACGGGTCTGTCTACTGGTCCTCCCAAGCCGAATCTCTGTTAGAGCAA
      S  C  Q  Q  H  G  S  V  Y  W  S  S  Q  A  E  S  L  L  E  Q

721  TTGAATAAGAAACCGGAAAAAGATTTATTGCACTACGTGAGCTCTGTAGCATTTGCAAGG
      L  N  K  K  P  E  K  D  L  L  H  Y  V  S  S  V  A  F  A  R

781  CAAACCGCCCCCAATTGGCAAGACCACAGTACTGCAAAGCAATGTCAAGAGTTTGAAGAG
      Q  T  A  P  N  W  Q  D  H  S  T  A  K  Q  C  Q  E  F  E  E

841  TGCATAGGTGGGCCTGAAAAAATGGCTCAATTAACAGGGTCCAGAGCCCATTTTAGATTT
      C  I  G  G  P  E  K  M  A  Q  L  T  G  S  R  A  H  F  R  F

901  ACTGGTCCTCAAATTCTGAAAATTGCACAATTAGAACCAGAAGCTTACGAAAAAACAAAG
      T  G  P  Q  I  L  K  I  A  Q  L  E  P  E  A  Y  E  K  T  K

961  ACCATTTCTTTAGTGTCTAATTTTTTGACTTCTATCTTAGTGGGCCATCTTGTTGAATTA
      T  I  S  L  V  S  N  F  L  T  S  I  L  V  G  H  L  V  E  L

1021 GAGGAGGCAGATGCCTGTGGTATGAACCTTTATGATATACGTGAAAGAAAATTCATGTAT
      E  E  A  D  A  C  G  M  N  L  Y  D  I  R  E  R  K  F  M  Y

1081 GAGCTACTACATCTAATTGATAGTTCTTCTAAGGATAAAACTATCAGACAAAAATTAATG
      E  L  L  H  L  I  D  S  S  S  K  D  K  T  I  R  Q  K  L  M

1141 AGAGCACCCATGAAAAATTTGATAGCGGGTACCATCTGTAAATATTTTATTGAGAAGTAC
      R  A  P  M  K  N  L  I  A  G  T  I  C  K  Y  F  I  E  K  Y

1201 GGTTTCAATACAAACTGCAAGGTCTCTCCCATGACTGGGGATAATTTAGCCACTATATGT
      G  F  N  T  N  C  K  V  S  P  M  T  G  D  N  L  A  T  I  C

1261 TCTTTACCCCTGCGGAAGAATGACGTTCTCGTTTCCCTAGGAACAAGTACTACAGTTCTT
      S  L  P  L  R  K  N  D  V  L  V  S  L  G  T  S  T  T  V  L

1321 CTGGTCACCGATAAGTATCACCCCTCTCCGAACTATCATCTTTTCATTCATCCAACTCTG
      L  V  T  D  K  Y  H  P  S  P  N  Y  H  L  F  I  H  P  T  L

1381 CCAAACCATTATATGGGTATGATTTGTTATTGTAATGGTTCTTTGGCAAGGGAGAGGATA
      P  N  H  Y  M  G  M  I  C  Y  C  N  G  S  L  A  R  E  R  I
```

Figure 2 (1/2)

```
1441  AGAGACGAGTTAAACAAAGAACGGGAAAATAATTATGAGAAGACTAACGATTGGACTCTT
        R  D  E  L  N  K  E  R  E  N  N  Y  E  K  T  N  D  W  T  L
1501  TTTAATCAAGCTGTGCTAGATGACTCAGAAAGTAGTGAAAATGAATTAGGTGTATATTTT
        F  N  Q  A  V  L  D  D  S  E  S  S  E  N  E  L  G  V  Y  F
1561  CCTCTGGGGGAGATCGTTCCTAGCGTAAAAGCCATAAACAAAAGGGTTATCTTCAATCCA
        P  L  G  E  I  V  P  S  V  K  A  I  N  K  R  V  I  F  N  P
1621  AAAACGGGTATGATTGAAAGAGAGGTGGCCAAGTTCAAAGACAAGAGGCACGATGCCAAA
        K  T  G  M  I  E  R  E  V  A  K  F  K  D  K  R  H  D  A  K
1681  AATATTGTAGAATCACAGGCTTTAAGTTGCAGGGTAAGAATATCTCCCCTGCTTTCGGAT
        N  I  V  E  S  Q  A  L  S  C  R  V  R  I  S  P  L  L  S  D
1741  TCAAACGCAAGCTCACAACAGAGACTGAACGAAGATACAATCGTGAAGTTTGATTACGAT
        S  N  A  S  S  Q  Q  R  L  N  E  D  T  I  V  K  F  D  Y  D
1801  GAATCTCCGCTGCGGGACTACCTAAATAAAAGGCCAGAAAGGACTTTTTTTGTAGGTGGG
        E  S  P  L  R  D  Y  L  N  K  R  P  E  R  T  F  F  V  G  G
1861  GCTTCTAAAAACGATGCTATTGTGAAGAAGTTTGCTCAAGTCATTGGTGCTACAAAGGGT
        A  S  K  N  D  A  I  V  K  K  F  A  Q  V  I  G  A  T  K  G
1921  AATTTTAGGCTAGAAACACCAAACTCATGTGCCCTTGGTGGTTGTTATAAGGCCATGTGG
        N  F  R  L  E  T  P  N  S  C  A  L  G  G  C  Y  K  A  M  W
1981  TCATTGTTATATGACTCTAATAAAATTGCAGTTCCTTTTGATAAATTTCTGAATGACAAT
        S  L  L  Y  D  S  N  K  I  A  V  P  F  D  K  F  L  N  D  N
2041  TTTCCATGGCATGTAATGGAAAGCATATCCGATGTGGATAATGAAAATTGGATCGCTATA
        F  P  W  H  V  M  E  S  I  S  D  V  D  N  E  N  W  I  A  I
2101  ATTCCAAGATTGTCCCCTTAAGCGAACTGGAAAAGACTCTCATCTAAAATATGTTTGAAT
        I  P  R  L  S  P
2161  AATTTATCATGCCCTGACAAGTACACACAAACACAGACACATAATATACATACATATATA

2221  TATATCACCGTTATTATGCGTGCACATGACAATGCCCTTGTATGTTTCGTATACTGTAGC

2281  AAGTAGTCATCATTTTGTTCCCCGTTCGGAAAATGACAAAAAGTAAAATCAATAAATGAA

2341  GAGTAAAAAACAATTTATGAAAGGGTGAGCGACCAGCAACGAGAGAGACAAATCAAATTA

2401  GCGCTTTCCAGTGAGAATATAAGAGAGCATTGAAAGAGCTAGGTTATTGTTAAATCATCT

2461  CGAGCTC
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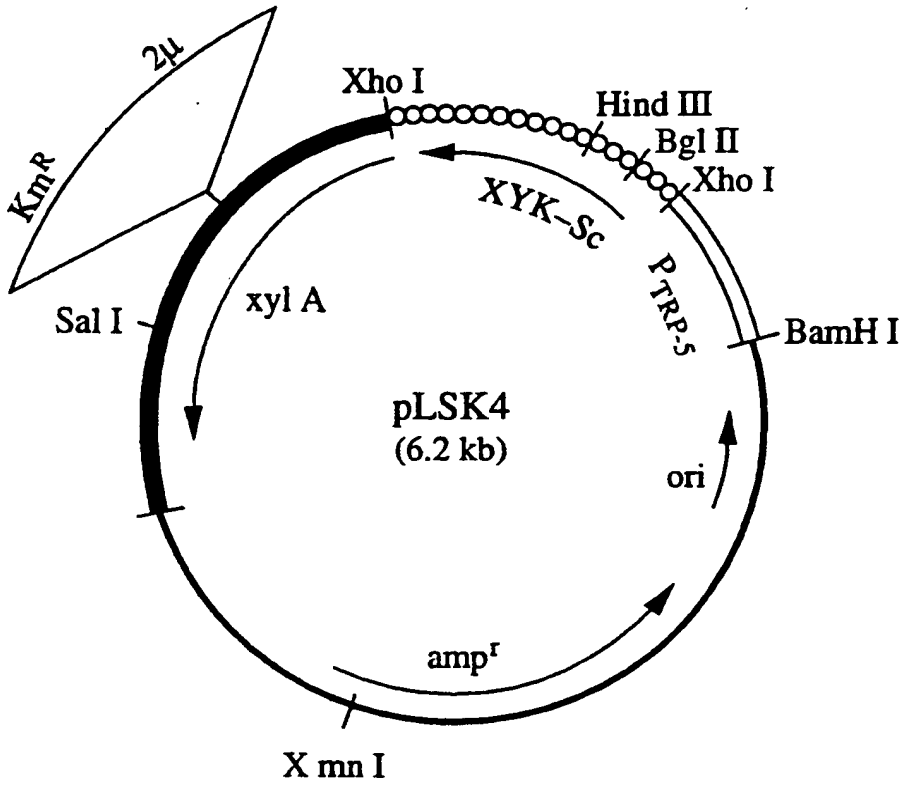
Figure 2 (2/2)



pLSK15

Figure 3.

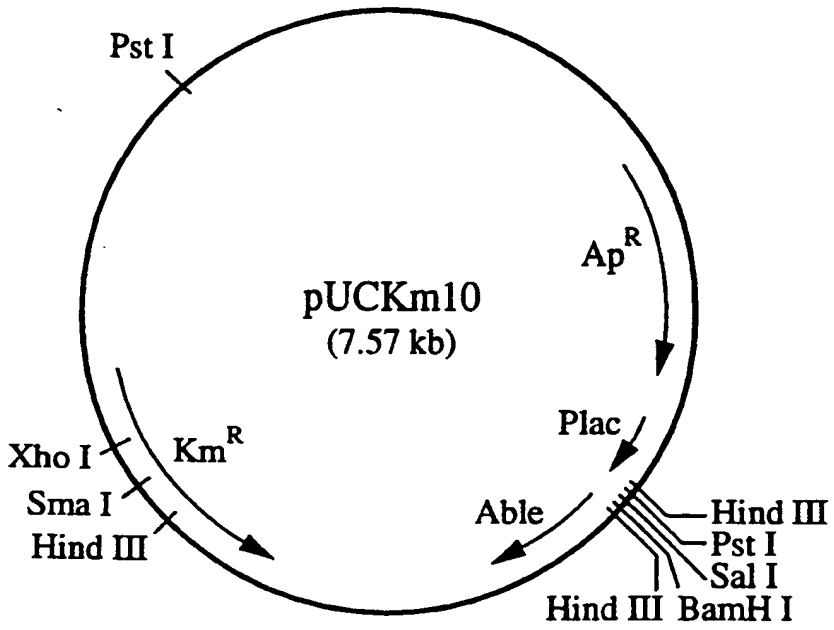
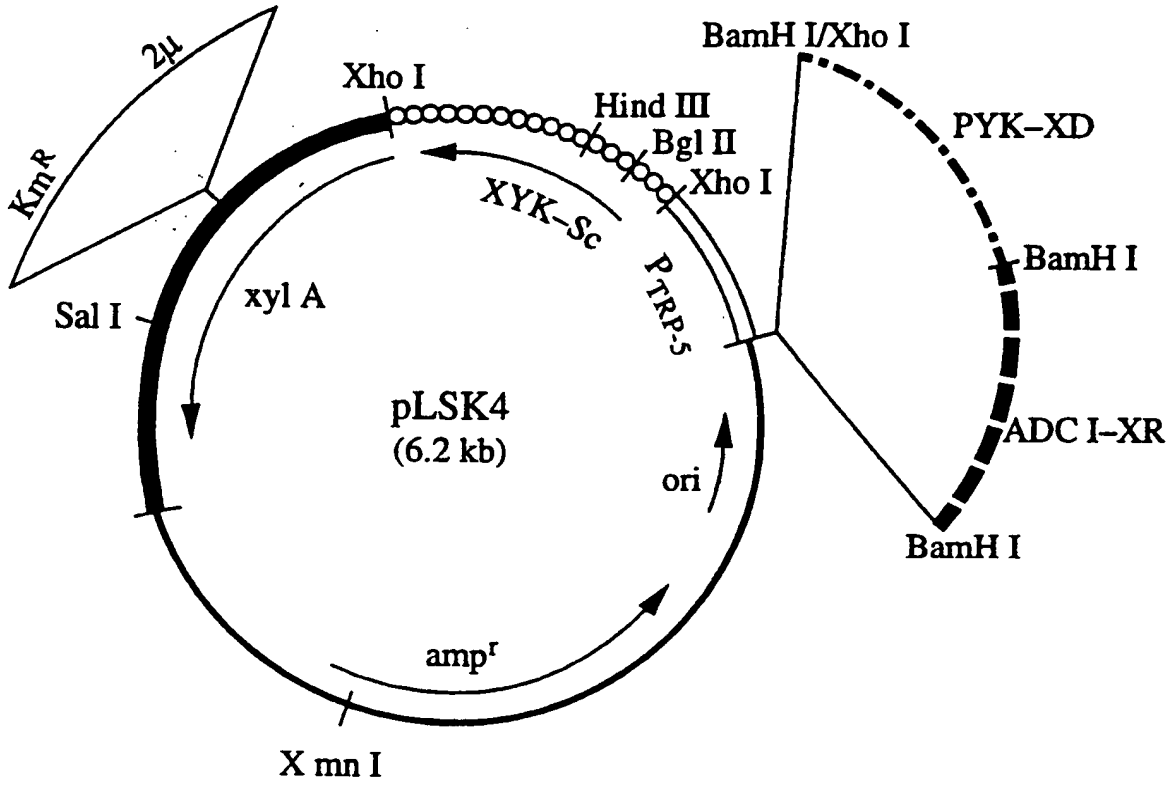

Pst I
Ap^R
pUCKm10
(7.57 kb)
Plac
Xho I
Km^R
Sma I
Hind III
Able
Hind III
Pst I
Sal I
Hind III BamH I

Figure 4.



pLNH21

Figure 5.

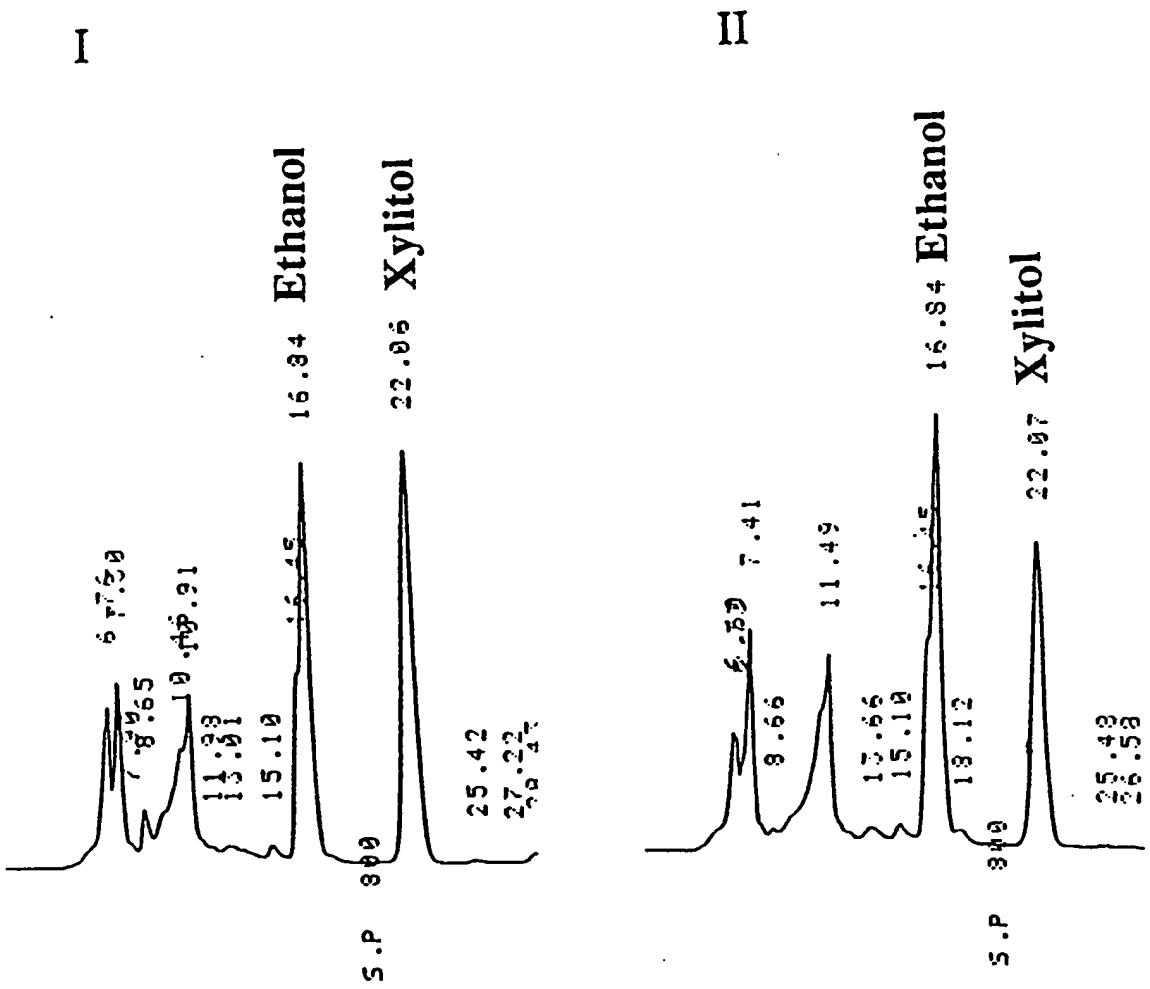


Figure 6A

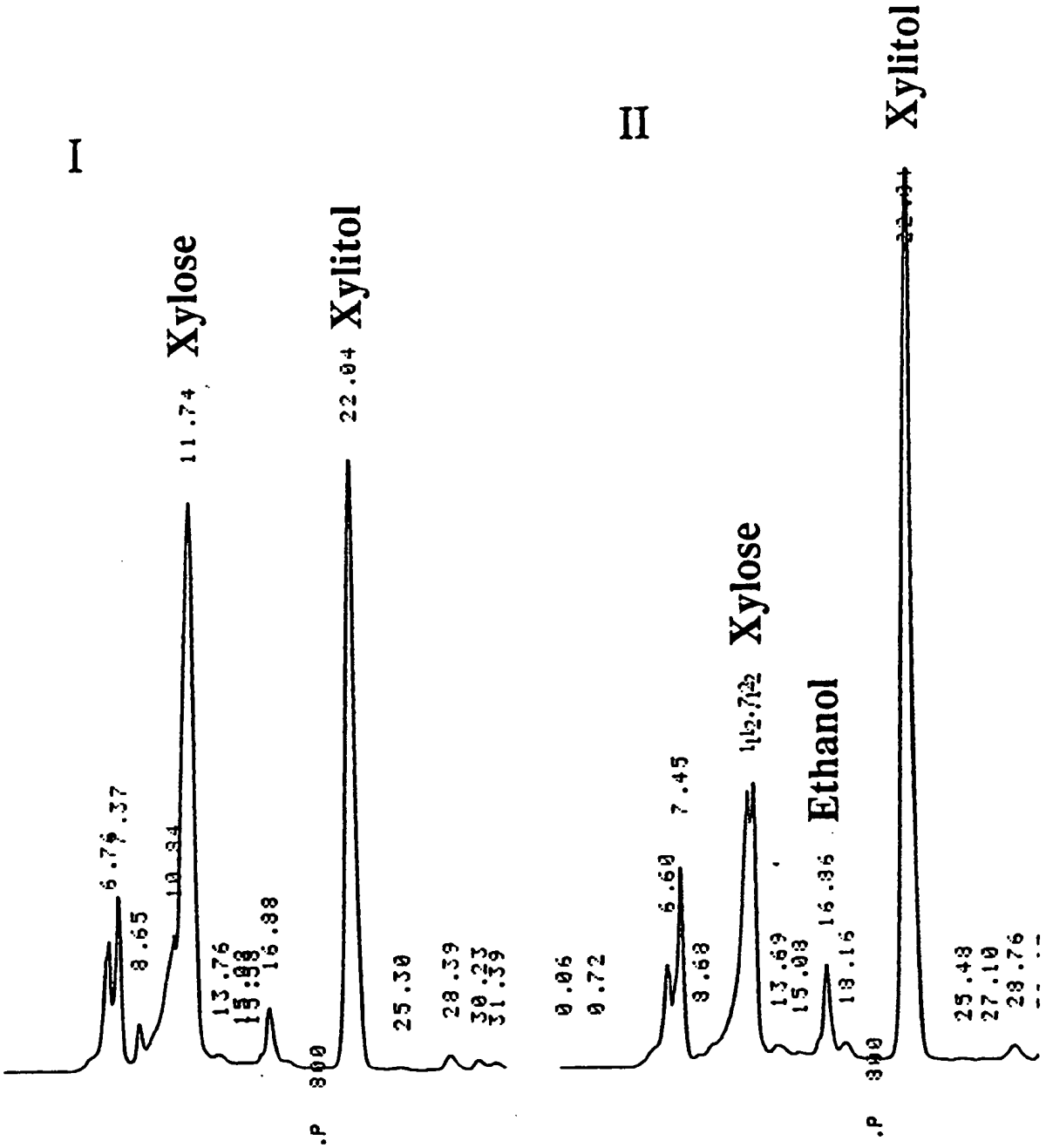

I
Xylose
Xylitol
6.79
7.37
8.65
10.34
11.74
13.76
15.08
15.88
22.04
25.30
28.39
30.23
31.39
II
Xylose
Ethanol
Xylitol
6.60
7.45
8.68
12.72
13.69
15.03
16.36
18.16
25.43
27.10
28.76

Figure 6B

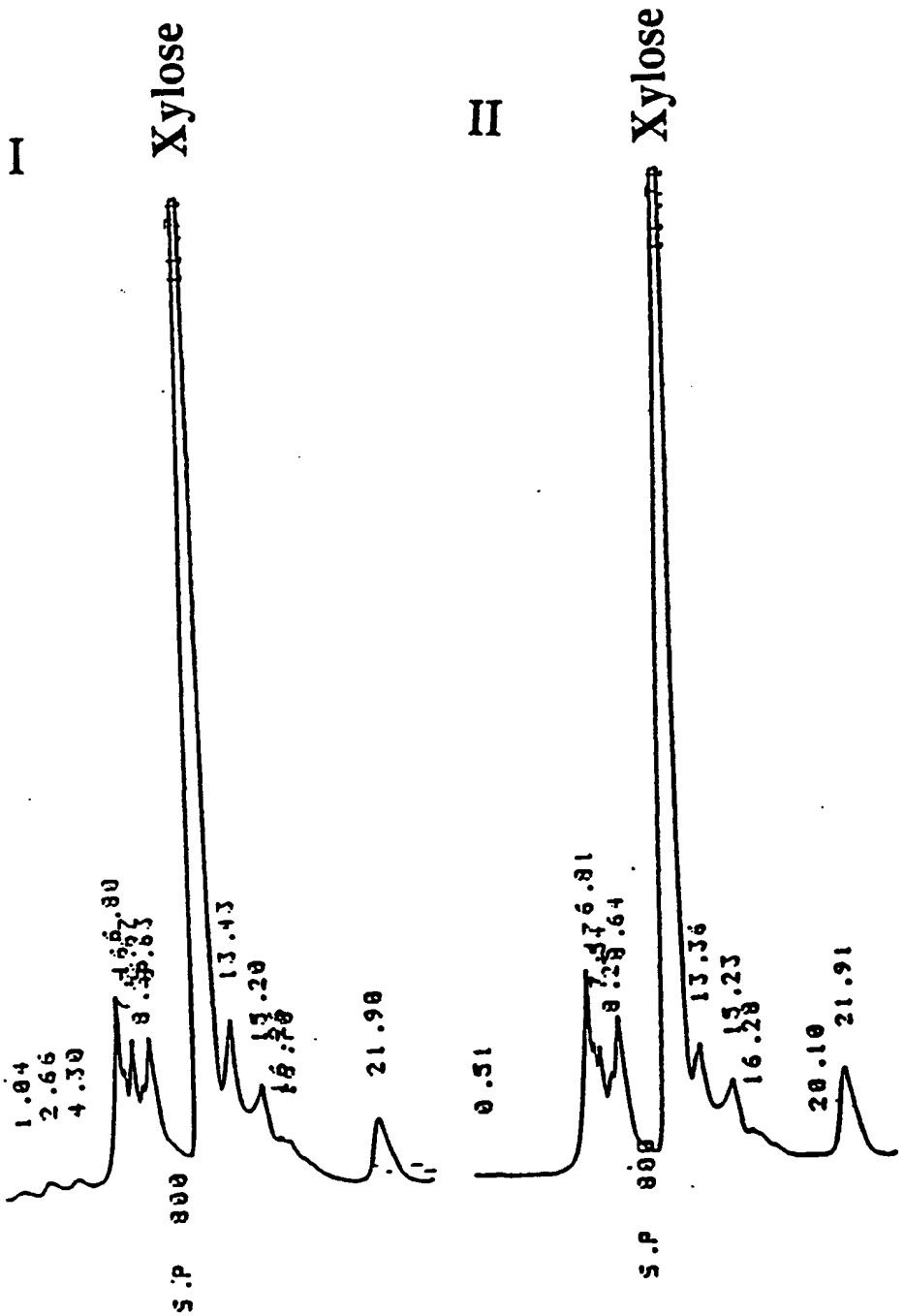
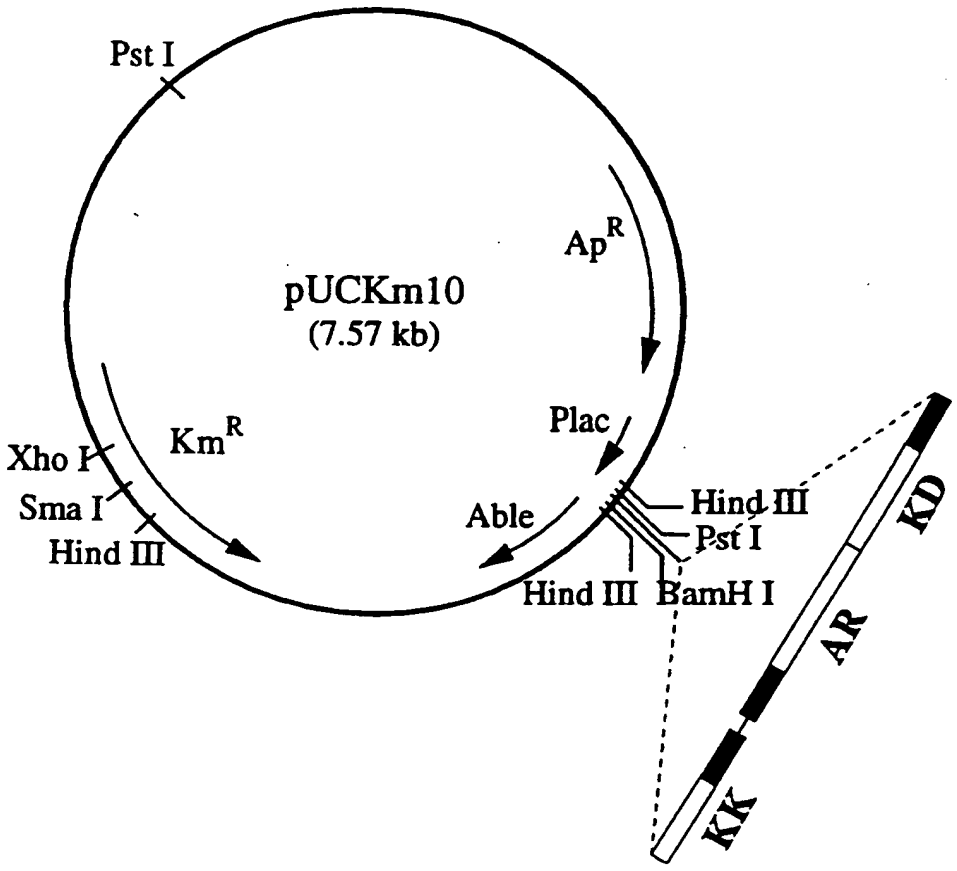

I
Xylose
1.04
2.66
4.30
13.43
13.20
21.98
S.P
II
Xylose
0.51
13.36
16.23
20.10
21.91
S.P

Figure 6C



pLNH33

Figure 7.

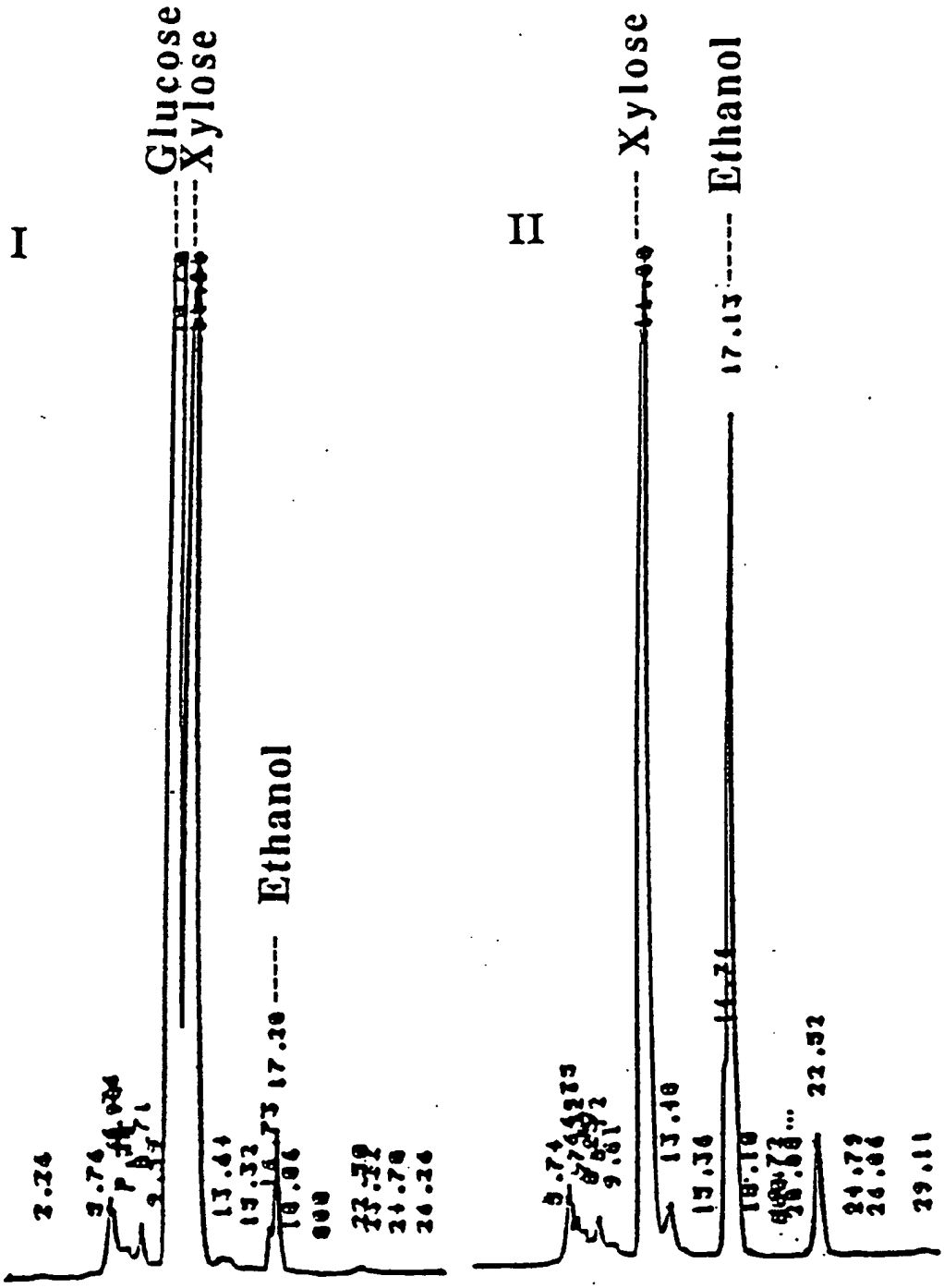


Figure 8A

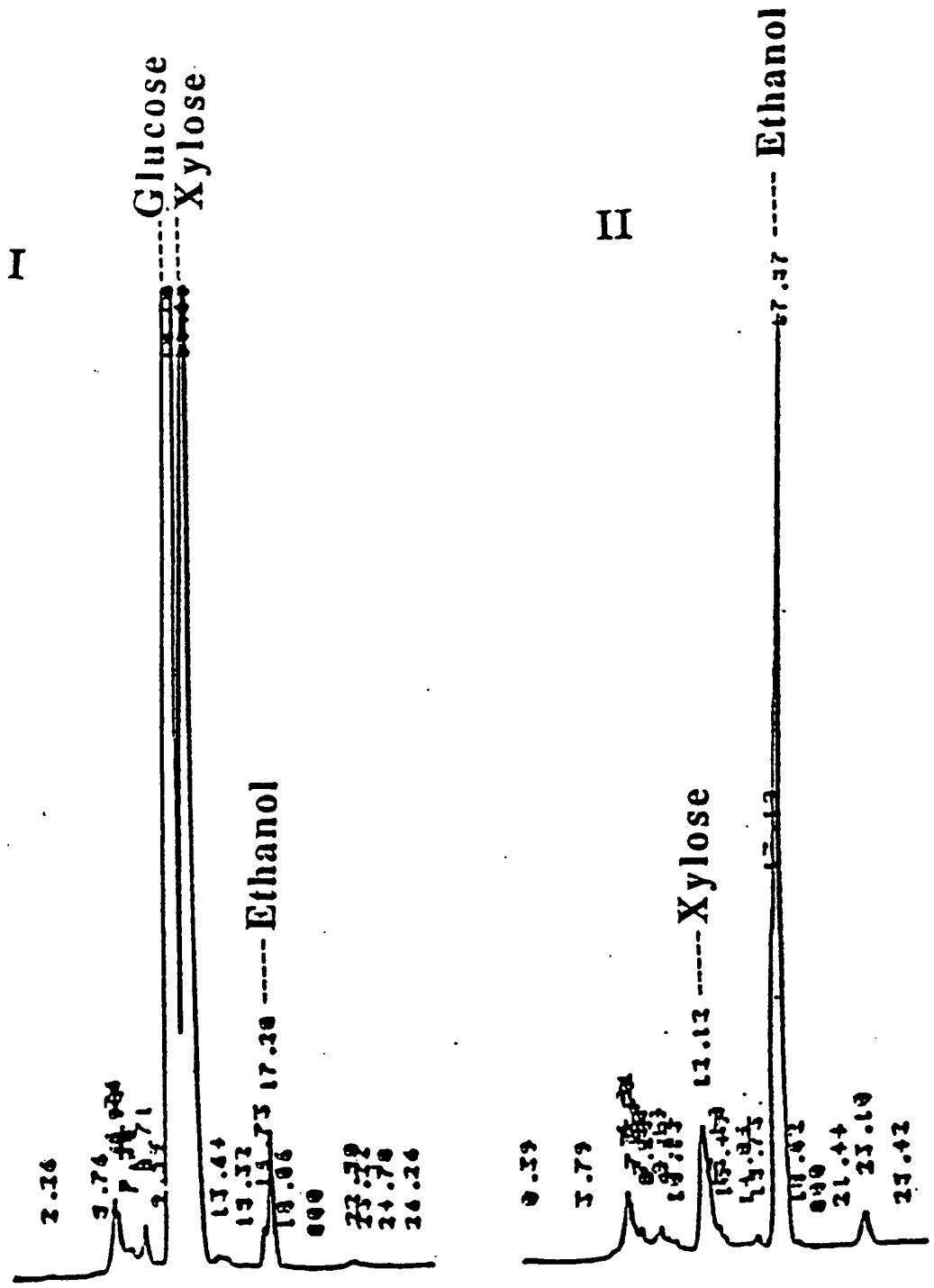


Figure 8B

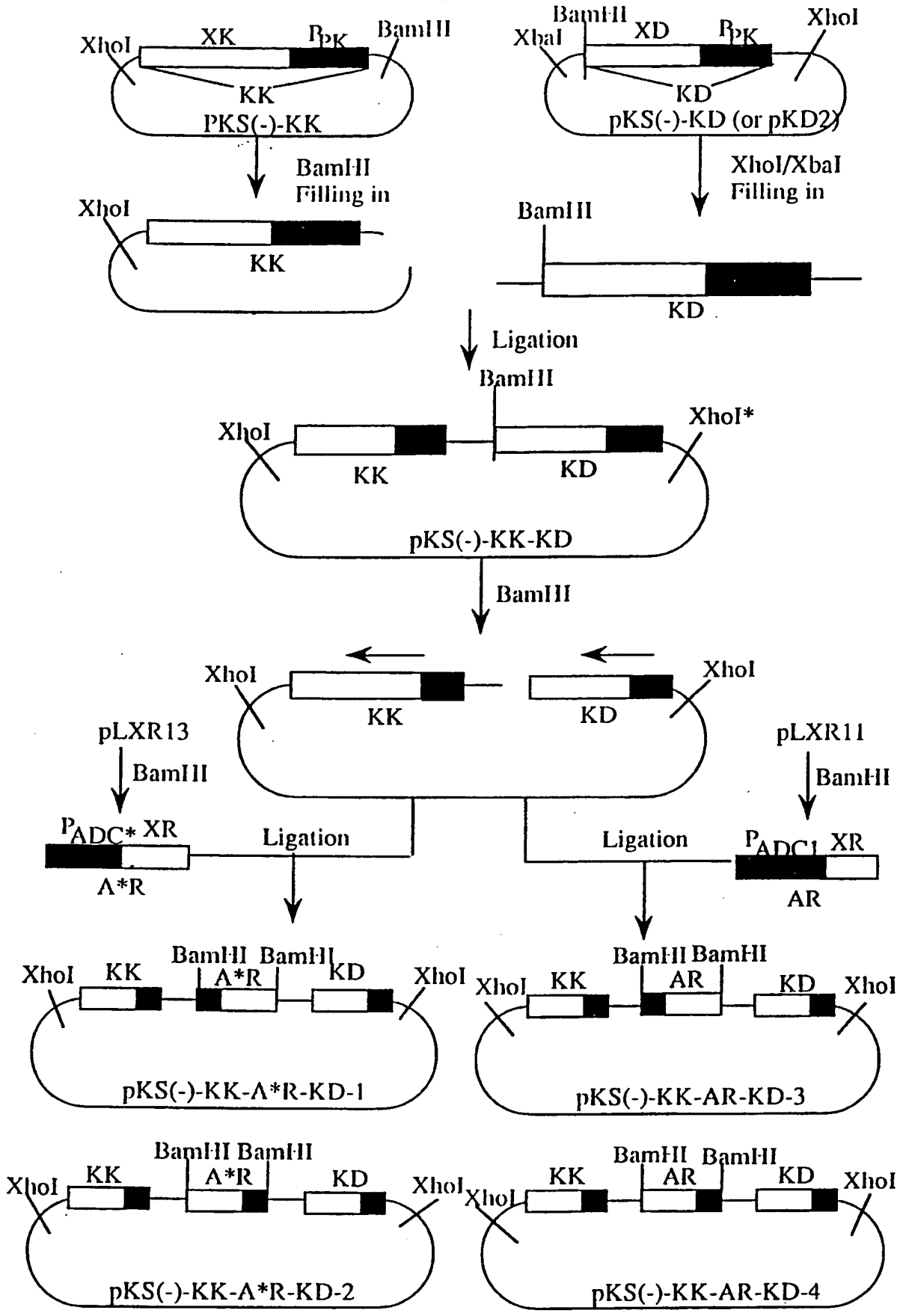


Figure 9. Construction of pKS(-)-KK-AR-KD plasmids
*The XhoI site was regenerated after ligation.

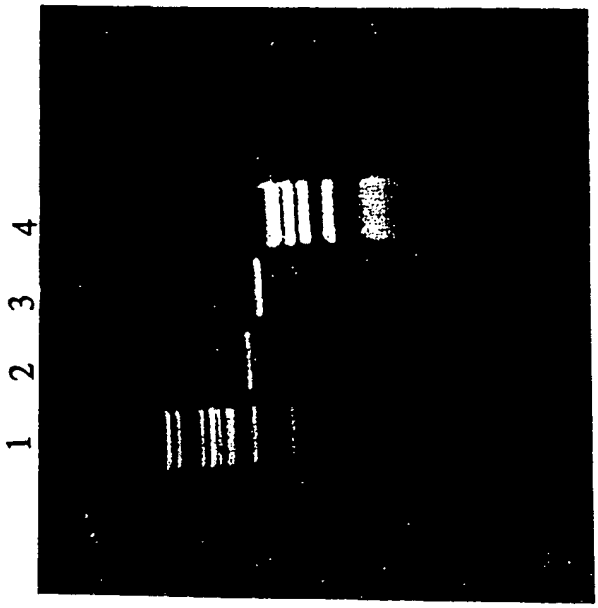


Figure 10. Direct amplification of the intact xylitol dehydrogenase gene (XD) and the promoterless XD from *Pichia stipitis* chromosomal DNA by polymerase chain reaction (PCR) technique.

1. Molecular markers BamHI-EcoRI digested λ DNA.
2. *Pichia* xylitol dehydrogenase gene (intact).
3. *Pichia* xylitol dehydrogenase gene (promoterless).
4. Molecular markers, Hae III digested ϕ X DNA.

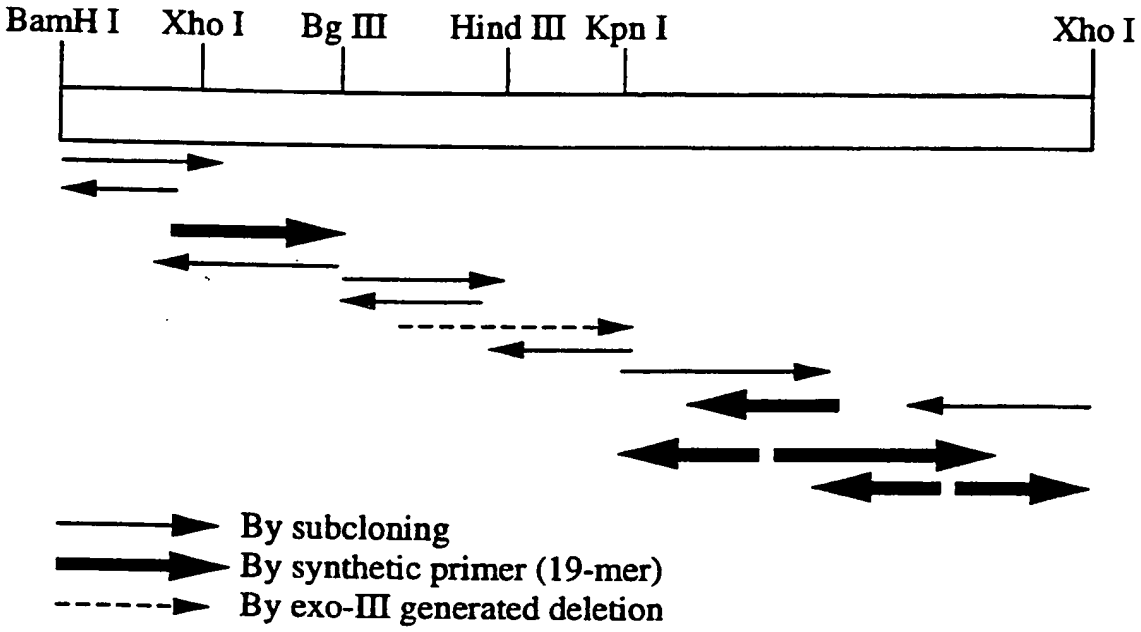

BamH I
Xho I
Bg III
Hind III
Kpn I
Xho I
By subcloning
By synthetic primer (19-mer)
By exo-III generated deletion

Figure 11.

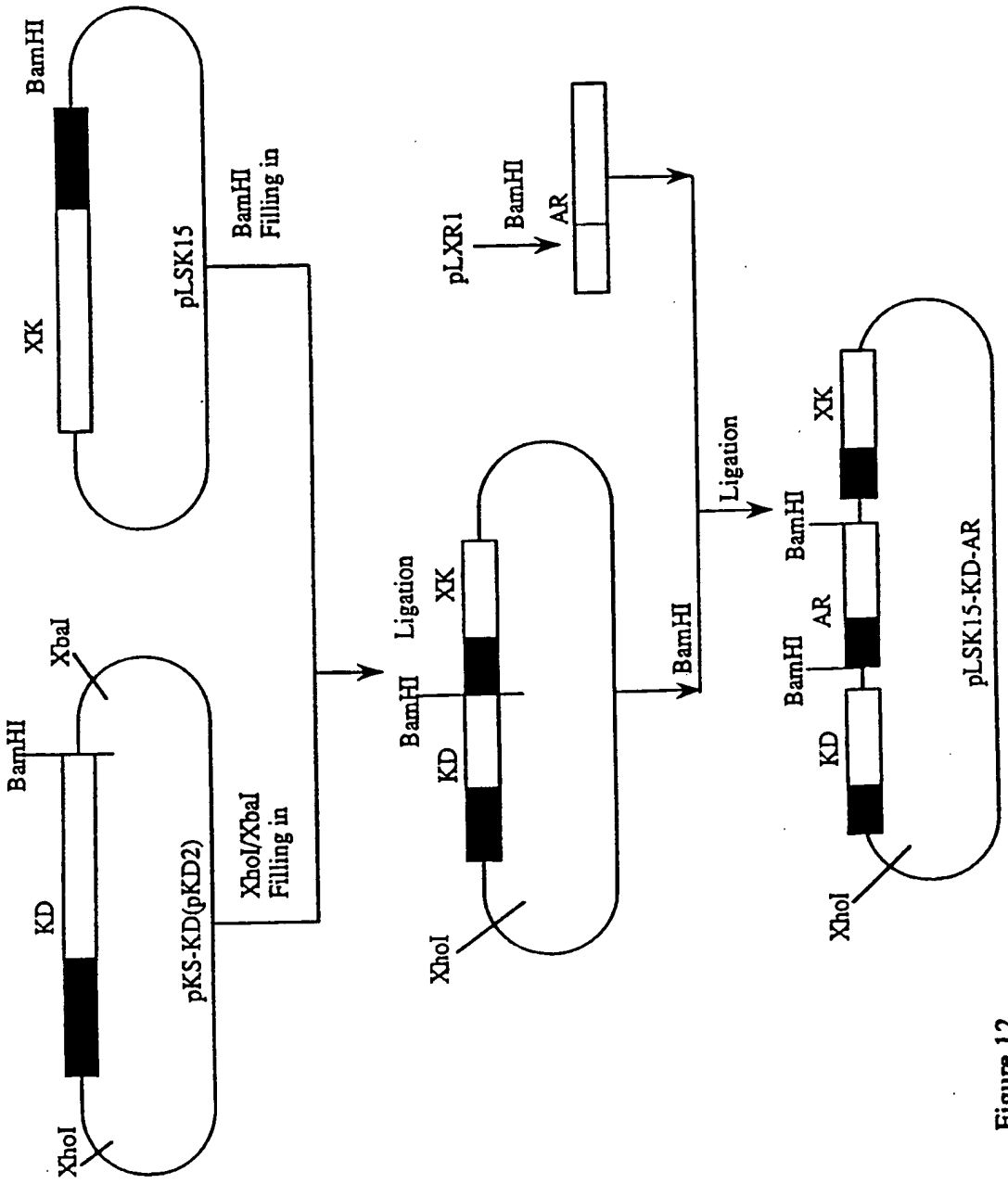

BamHI
XK
pLSK15
BamHI
Filling in
XbaI
BamHI
KD
pKS-KD(pKD2)
XhoI
XhoI/XbaI
Filling in
Ligation
XK
BamHI
KD
XhoI
pLXR1
BamHI
AR
BamHI
Ligation
XK
BamHI
AR
BamHI
KD
XhoI
pLSK15-KD-AR

Figure 12

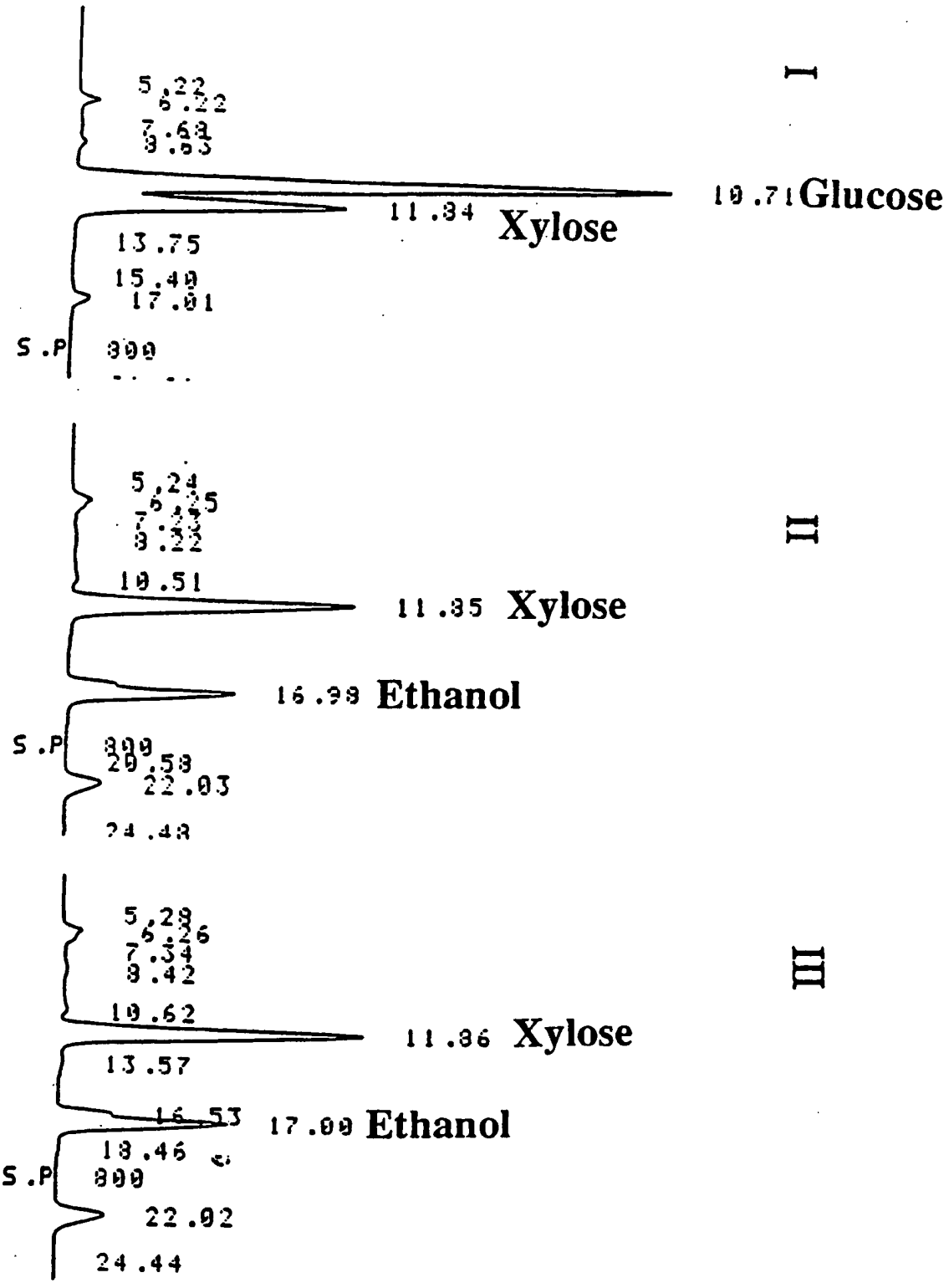

I
5.22
6.22
7.68
8.63
10.71 Glucose
11.84 Xylose
13.75
15.40
17.01
S.P 800
II
5.24
6.25
7.23
8.22
10.51
11.85 Xylose
16.98 Ethanol
S.P 800
20.58
22.03
24.48
III
5.28
6.26
7.34
8.42
10.62
11.86 Xylose
13.57
16.53
17.09 Ethanol
18.46
S.P 800
22.02
24.44

Figure 13

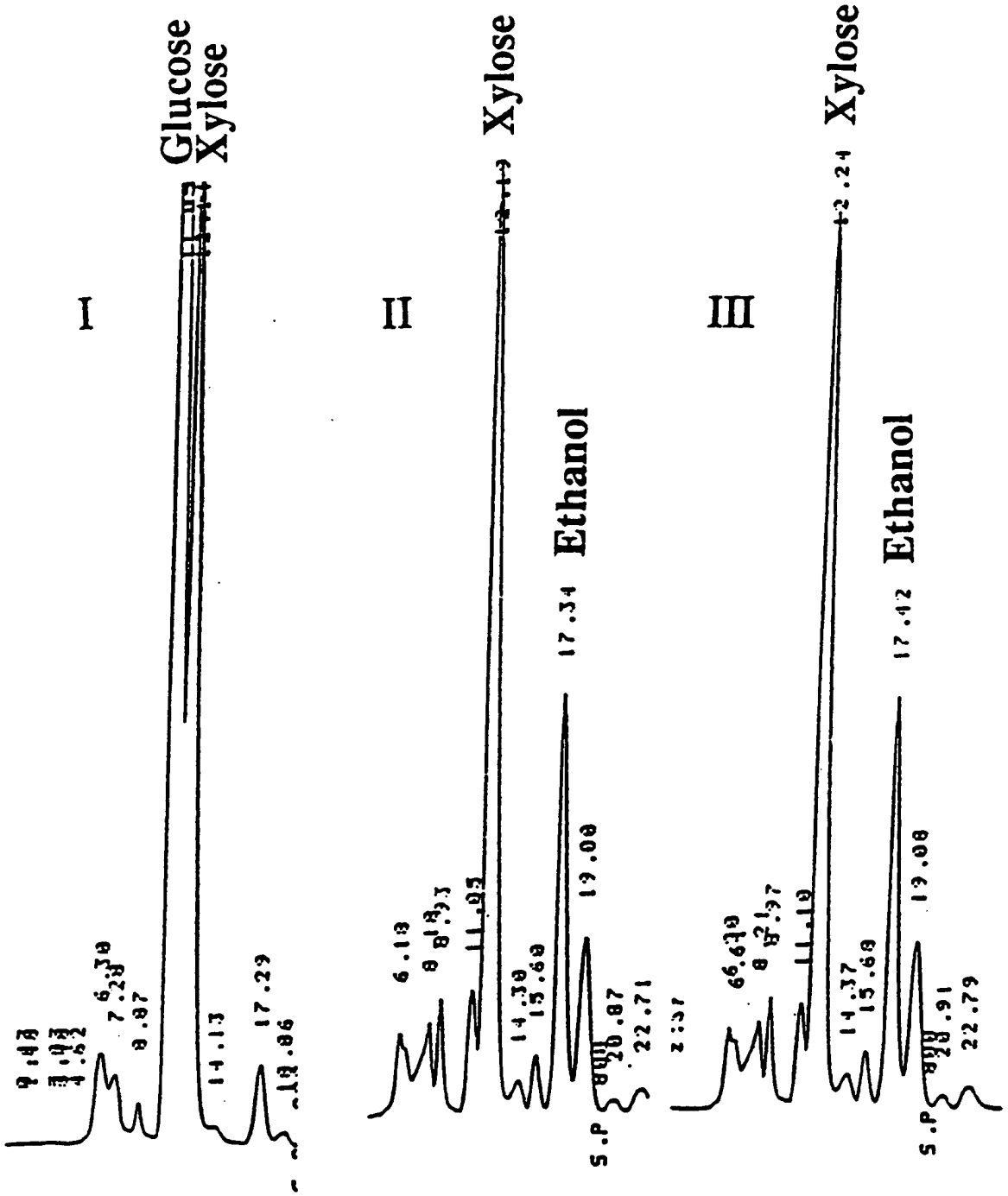

I
II
III
Glucose
Xylose
Xylose
Xylose
Ethanol
Ethanol
17.34
17.42
19.08
19.08
17.29
22.71
22.79
S.P
S.P

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12861

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 1/14, 9/00, 9/12, 15/00; C12P 7/08
US CL :435/163, 172.3, 183, 194, 254.21, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/163, 172.3, 183, 194, 254.21, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Applied Microbiology and Biotechnology, Volume 30, issued 1989, Amore et al., "The Fermentation of Xylose - an Analysis of the Expression of *Bacillus* and *Actinoplanes* Xylose Isomerase Genes in Yeast", pages 351-357, see entire document.	1-20
Y	The Journal of Biological Chemistry, Volume 258, Number 4, issued 25 February 1983, Burke et al., "The Isolation, Characterization, and Sequence of the Pyruvate Kinase Gene of *Saccharomyces cerevisiae*", pages 2193-2201, see entire document.	2-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Enzyme Microb. Technol., Volume 11, issued July 1989, Ho et al., "Cloning of Yeast Xylulokinase Gene by Complimentation of *E. coli* and Yeast Mutations", pages 417-421, see entire document.	1-20
Y	Current Genetics, Volume 18, issued 1990, "Isolation and Characterization of the *Pichia stipitis* Xylitol Dehydrogenase Gene, *XYL2*, and Construction of a Xylose-Utilizing *Saccharomyces cerevisiae* Transformant", pages 493-500, see entire document.	1-20

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(54) Titre : LEVURES RECOMBINANTES CONTENANT LES SEQUENCES D'ADN ENCODANT LA XYLOSE-REDUCTASE ET LA XYLITOL-DESHYDROGENASE

(54) Title: RECOMBINANT YEASTS CONTAINING THE DNA SEQUENCES CODING FOR XYLOSE REDUCTASE-AND XYLITOL DEHYDROGENASE ENZYMES

(57) Abrégé/Abstract:
This invention relates to recombinant-DNA-technology. Specifically this invention relates to new recombinant yeast strains transformed with xylose reductase and/or xylitol dehydrogenase enzyme genes. A yeast strain transformed with the xylose reductase gene is capable of reducing xylose to xylitol and consequently of producing xylitol in vivo. If both of these genes are transformed into a yeast strain, the resultant strain is capable of producing ethanol on xylose containing medium during fermentation. Further, the said new yeast strains are capable of expressing the said two enzymes. Xylose reductase produced by these strains can be used in an enzymatic process for the production of xylitol in vitro.

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(54) Title: RECOMBINANT YEASTS CONTAINING THE DNA SEQUENCES CODING FOR XYLOSE REDUCTASE- AND XYLITOL DEHYDROGENASE ENZYMES

(57) Abstract

This invention relates to recombinant-DNA-technology. Specifically this invention relates to new recombinant yeast strains transformed with xylose reductase and/or xylitol dehydrogenase enzyme genes. A yeast strain transformed with the xylose reductase gene is capable of reducing xylose to xylitol and consequently of producing xylitol *in vivo*. If both of these genes are transformed into a yeast strain, the resultant strain is capable of producing ethanol on xylose containing medium during fermentation. Further, the said new yeast strains are capable of expressing the said two enzymes. Xylose reductase produced by these strains can be used in an enzymatic process for the production of xylitol *in vitro*.

RECOMBINANT YEASTS CONTAINING THE DNA SEQUENCES CODING FOR XYLOSE REDUCTASE- AND XYLITOL DEHYDROGENASE ENZYMES.

Field of the invention

This invention relates to recombinant-DNA-technology. Specifically this invention relates to new recombinant yeast strains transformed with xylose reductase and/or xylitol dehydrogenase enzyme genes. A yeast strain transformed with the xylose reductase gene is capable of reducing xylose to xylitol and consequently of producing xylitol *in vivo*. If both of these genes are transformed into a yeast strain, the resultant strain is capable of producing ethanol on xylose containing medium during fermentation.

Further, the said new yeast strains are capable of expressing the said two enzymes. Xylose reductase produced by these strains can be used in an enzymatic process for the production of xylitol *in vitro*.

Background of the invention

Xylose utilization

Xylose appears in great abundance in nature. It can constitute as much as 40 % of a lignocellulosic material (Ladisch *et al.*, 1983). By fermentation xylose can be converted to ethanol which can be used as a liquid fuel or a chemical feedstock. Enzymatically or as a by-product of fermentation xylose can also be converted to xylitol which is a promising natural sweetener having dental caries reducing properties. Xylitol can also be used by diabetics. For the production of ethanol which is a cheap product it is important that the raw material can be fermented directly with as little pretreatment as possible. For the production of xylitol which is meant for human consumption it is important that the process involves GRAS (Generally Recognized As Safe) organisms.

Natural xylose utilizers are found among bacteria, yeast and fungi. In all organisms xylose is converted to xylulose which is phosphorylated to xylulose-5-phosphate (X5P) with xylulokinase. X5P then enters the Embden-Meyerhof pathway (glycolysis) via the pentose phosphate shunt.

Bacteria like *Escherichia coli*, *Bacillus* sp., *Streptomyces* sp. and *Actinoplanes* sp. convert xylose directly to xylulose with a xylose isomerase (XI). Thus bacteria do not produce xylitol as an intermediate during xylose utilization. Those which ferment xylose to ethanol do so with poor yields because a number of by-products are also produced (Skoog and Hahn-Hägerdal, 1988). In xylose utilizing yeasts such as *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* this reaction occurs in two steps: first xylose is reduced to xylitol with a xylose reductase (XR) and the xylitol is oxidized with a xylitol dehydrogenase (XDH) to xylulose.

Pure xylose solutions are fermented with high yields and good productivities by xylose utilizing yeasts such as *P. stipitis*, *C. shehatae* and *P. tannophilus* (Slininger *et al.*, 1987; Prior *et al.*, 1989). However, they do not generally survive in the hostile environment of an untreated raw material such as eg. spent sulphite liquor or hydrogen fluoride-pretreated and acid-hydrolyzed wheat straw (Lindén and Hahn-Hägerdal, 1989,a, b). The one exception, *P. tannophilus*, produces mainly xylitol and glycerol in response to this environment. In order to efficiently ferment such raw materials with the xylose utilizing yeasts such as *P. stipitis*, *C. shehatae* and *P. tannophilus* the raw material has to undergo expensive pretreatments with ion-exchange resins (Clark and Mackie, 1984) or steam stripping (Yu *et al.*, 1987).

Saccharomyces cerevisiae, bakers' yeast, ferments spent sulphite liquor or hydrogen fluoride-pretreated and acid-hydrolyzed wheat straw to ethanol (Lindén and Hahn-Hägerdal, 1989). *S. cerevisiae* cannot utilize xylose efficiently and cannot grow on xylose as a sole carbon source. In the presence of the bacterial enzyme xylose isomerase, which converts xylose to xylulose, *S. cerevisiae* can, however, ferment both pure xylose solutions (Hahn-Hägerdal *et al.*, 1986) and untreated raw materials (Lindén and Hahn-Hägerdal, 1989a,b) to ethanol with yields and productivities that are in the same order of magnitude as those obtained in hexose fermentations. Similar results have been obtained with *Schizosaccharomyces pombe* (Lastick *et al.*, 1989). Thus, both *S. cerevisiae* and *Sch. pombe* have a functioning xylulokinase enzyme. It has also been found that *S. cerevisiae* can take up xylose (Batt *et al.*, 1986; van Zyl *et al.*, 1989; Senac and Hahn-Hägerdal, 1990).

Gong (1985) discloses a process for obtaining ethanol directly from D-xylose by xylose fermenting yeast mutants. According to Gong a parent yeast strain is selected (e.g. *Candida* sp. or *Saccharomyces cerevisiae*), which originally may have the ability to utilize D-xylose, and this parent strain is then exposed e.g. to UV-radiation so as to induce mutation. However, no information about the reason why the mutants obtained are able to utilize xylose, is given in the reference. Further, Gong did not introduce any new coding and/or regulatory sequences to said strains by genetic engineering techniques to enhance xylose fermentation.

Xylitol is industrially manufactured at the moment by chemical reduction of hemicellulose hydrolysates. Poisoning of the expensive catalyst used in the reduction step and formation of side-products difficult to be separated from the end product are the main problems in this process.

In the literature there are numerous examples of microbiological methods to produce xylitol from pure xylose (eg. Onishi and Suzuki, 1966; Barbosa *et al.*, 1988). Best producers in this method are yeasts especially belonging to the *Candida*-genera. Also some bacteria such as *Enterobacter* (Yoshitake *et al.*, 1973a) and *Corynebacterium* species (Yoshitake *et al.*, 1973b) and some molds eg. *Penicillium chrysogenum* (Chiang and Knight, 1960) produce xylitol from pure xylose.

In a microbiological method describing the best yields of xylitol production (Ojamo *et al.*, 1987) *Candida guilliermondii* yeast is cultivated under strictly controlled aeration in a xylose containing medium either as a batch or a fed-batch process. Xylitol yields 50-65 % were obtained. The yield could be increased to 76 % by adding furfuraldehyde to the cultivation medium.

Cell-free extracts from *Candida pelliculosa* (xylose reductase) and *Methanobacterium* sp. (hydrogenase, F_{420}, NADP, F_{420}/NADP oxidoreductase) has been used to produce xylitol in a membrane reactor with 90 % conversion (Kitpreechavanich, 1985). With a cell-free extract from a *Corynebacterium* species 69 % conversion has been obtained when 6-phosphogluconate was used for regeneration of the cofactor.

It has been shown that glucose dehydrogenase from *B. megaterium* has suitable

properties as a NADPH regenerating enzyme (Kulbe *et al.*, 1987). Thus gluconic acid from glucose can be produced simultaneously with xylitol in the enzymatic process. For the retention of the enzymes and the cofactor one can use ultrafiltration membranes. Cofactor retention may be achieved by the use of a derivatized cofactor having high enough molecular weight for the retention (Kulbe *et al.*, 1987) or better by using negatively charged ultrafiltration membranes (Kulbe *et al.*, 1989).

Attraction to use an enzymatic method is based on the possibility to use impure xylose containing raw materials which in the microbiological methods would inhibit the metabolism of the microbe used. Also the yields of xylitol are higher than in the microbiological methods with natural strains. On the other hand any microbiological method is more simple in large-scale practice at the moment.

The natural xylose utilizing yeasts such as *P. stipitis*, *Candida* sp. and *P. tannophilus* are not suitable for the production of either ethanol or xylitol for several reasons. The fermentation to ethanol requires pretreatment of the raw material which is cost-prohibitive for a cheap end-product such as ethanol. These species also lack the GRAS-status. Thus xylose utilization would most suitably be based on the use of bakers' yeast which has a GRAS-status.

In order to make *S. cerevisiae* an efficient xylose utilizer for the production of xylitol and ethanol an efficient enzyme system for the conversion of xylose to xylitol and xylulose should be introduced into this yeast. For the production of ethanol from xylose the XI genes from *E. coli* (Sarthy *et al.*, 1987), *B. subtilis* and *Actinoplanes missouriensis* (Amore *et al.*, 1989) have been cloned and transformed into *S. cerevisiae*. The XI protein made in *S. cerevisiae* had very low (1/1000 of the enzyme produced in bacteria) or no enzymatic activity. Thus, for some reasons the bacterial enzyme can not be made functional in yeast. Another possibility would be to transfer into *S. cerevisiae* the genes encoding XR and XDH from another yeast. The enzymes of *P. stipitis* should be good candidates in the light of the efficient utilization of pure xylose solutions discussed above. It can be anticipated that enzymes from another yeast would function better than bacterial enzymes when expressed in yeast. In addition, xylitol and ethanol could be produced with the same system and the system would combine the good xylose utilization of *P. stipitis* with resistance to inhibitors

and general acceptance of *S. cerevisiae*.

Summary of the invention

The present invention describes the isolation of genes coding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from certain yeasts having these genes, the characterization of the genes and their transfer into, and their expression in *Saccharomyces cerevisiae*.

This invention thus provides new recombinant yeast strains expressing xylose reductase and/or xylitol dehydrogenase enzymes.

The yeast strains according to the invention being transformed with the XR gene are capable of reducing xylose to xylitol *in vivo*. Xylose reductase produced by the new yeast strains according to the invention is also used in an enzymatic process for the production of xylitol *in vitro*.

The present invention further provides new yeast strains transformed with both of the above mentioned two genes. The coexpression of these genes renders the strain capable of fermenting xylose to ethanol from pure xylose solution or xylose containing solutions such as lignocellulosic hydrolyzates.

Brief description of the drawings

Fig. 1 shows the xylose reductase gene integrated into pMA91 and pRS305 resulting in plasmids pUA103 and pUA107, respectively.

Fig. 2 Activity plate assay of a XDH positive λ gt11 clone

Fig. 3 shows the picture of the plasmid pJHXDH50 carrying the *xdh* gene.

Fig. 4 shows a plate activity assay of a recombinant *S. cerevisiae* strain VTT-C-91181 producing XDH, on the original transformation plate (A) and as single colonies obtained from this transformant (B).

Fig. 5 shows the xylose reductase expression cassette flanked by ribosomal sequences integrated into BS+, generating the vector pJHXR22.

Fig. 6 shows Western analysis of XR produced in *S. cerevisiae* from the following plasmids and from *P. stipitis*. Lanes: 1 molecular weight standars (LMW, Pharmacia); 2 pRS305; 3 pUA107; 4 empty lane; 5 pMA91; 6 pUA103; 7 purified XR enzyme; 8 LMW; 9 *Pichia stipitis*. Samples were taken from French pressed cell lysates for lanes 2, 3, 5, 6 and 9.

Fig. 7 shows XR activity of *S. cerevisiae* strains transformed with pUA103 and pUA107 using as a cofactor either NADH or NADPH, and of the control strains carrying the vector pRS305 or pMA91.

Fig. 8 shows the xylitol dehydrogenase gene integrated into pKB102 generating plasmid pJHXDH60.

Detailed description of the invention

Xylose reductase (*xrd*) and xylitol dehydrogenase (*xdh*) genes to be used in this invention are isolated from a yeast containing these genes, eg. *Pichia stipitis*. Also other suitable yeasts and other fungi, such as *Pachysolen tannophilus*, *Kluyveromyces* spp., *Petromyces albertensis* and *Candida* spp. can be used.

The yeast to be transformed with these genes can be any suitable yeast, for xylitol production preferably having the GRAS-status, eg. any *Saccharomyces cerevisiae* yeast strain, (eg. DBY746, AH22, S150-2B, GPY55-15Bα, VTT-A-63015, VTT-A-85068, VTT-C-79093), any *Kluyveromyces* sp. or *Schizosaccharomyces pombe*. Transfer of the genes into these yeasts can be achieved, for instance, by using the conventional methods described for these organisms. It is to be noticed that, if wanted, also *Pichia* itself can be transformed with these genes in order to obtain increased or modulated expression of the genes. *Saccharomyces cerevisiae* strains are preferable for the purposes of this invention.

The DNA sequence coding for XR enzyme is isolated from *P. stipitis* by conventional methods. In the preferred embodiment, cDNA is synthesized from mRNA and cloned into λ gt11 vector. Using immuno screening with XR specific antibodies, the positive clone is isolated and subcloned into BS+ vector for sequencing. Gene encoding XR of *P. stipitis* can be cloned also by expression in *S. cerevisiae* because it does not contain any introns. Another possibility is the use of oligonucleotides, designed on the basis of the amino acid sequence of the enzyme, in hybridization of a gene bank.

To construct a plasmid suitable for transformation into a yeast, the gene coding for XR is cloned into a suitable yeast expression vector, such as pMA91 (Mellor *et al.*, 1983), comprising the appropriate yeast regulatory regions. These regulatory regions can be obtained from yeast genes such as the *PGK1*, *ADH1*, *GAL1*, *GAL10*, *CUP1*, *GAP*, *CYC1*, *PHO5*, for instance. Alternatively, also the regulatory regions of the *Pichia* gene encoding XR can be used to express the gene in *S. cerevisiae*. The plasmid carrying the *xrd* gene encoding XR is capable of replicating autonomously when transformed into the recipient yeast strain. The gene encoding XR together with the appropriate yeast regulatory regions can also be cloned into a single copy yeast vector such as pRS305 (Sikorski and Hieter, 1989).

Alternatively, the gene coding for XR can also be integrated into the yeast chromosome, into the ribosomal RNA locus, for instance. For this purpose the ribosomal sequences of a suitable plasmid, eg. plasmid pIRL9 are released, and cloned appropriately to BS+ vector. The gene coding for XR, coupled in between suitable yeast promoter and terminator regions, is released from the hybrid vector comprising the gene and cloned into the plasmid obtained at the previous stage. From this resulting plasmid the expression cassette, flanked by ribosomal sequences can be released. This fragment is cotransformed into a yeast with an autonomously replicating plasmid carrying a suitable marker for transformation. The plasmid can be later on removed from the cells containing the *xrd* gene integrated in the chromosome by cultivating the cells in non-selective conditions. This way, recombinant strains can be obtained which carry no extra foreign DNA such as bacterial vector sequences. If a polyploid yeast strain, such as VTT-A-63015, is used the gene can be integrated also to an essential locus such as the *PGK1* or the *ADH1* locus.

An object of this invention is thus to provide the specific xylose reductase gene. The sequence of the *xrd* gene can be determined from the plasmids carrying it by using eg. the double stranded dideoxy nucleotide sequencing method (Zagursky *et al.*, 1986). The sequence of the *xrd* gene encoding XR of *P. stipitis* is given as the SEQ ID NO. 2.

Another object of this invention is to provide specific yeast vectors comprising the *xrd* gene. Such a vector is either an autonomously replicating multicopy or a single copy plasmid or a vector capable of integrating into the yeast chromosome, as described above.

Still another object of this invention is to provide yeast strains which comprise the DNA sequence coding for XR and are capable of expressing this enzyme.

Thus a process for producing xylose reductase enzyme is also provided. This process comprises:

(a) isolating the DNA sequence coding for xylose reductase from a suitable donor organism;

(b) constructing a yeast vector carrying said DNA sequence;

(c) transforming the vector obtained into a suitable yeast host to obtain a recombinant host strain;

(d) cultivating said recombinant host strain under conditions permitting expression of said xylose reductase; and

(e) recovering said xylose reductase.

Enzymes from organisms having a GRAS status are preferred in an enzymatic production of xylitol. Xylose reductase from *Pichia stipitis* have excellent enzymatical properties such as kinetic constants and stability without special stabilizing agents such as thiol-protecting chemicals. Production of this enzyme is now possible in a yeast having a GRAS status. The transformed yeast cells are cultivated in an appropriate medium. As the cultivation medium, a cheap medium such as one based on molasses can be used as xylose is not needed for induction as in naturally xylose utilizing yeasts. Yeast with intracellular xylose reductase is produced with a good

yield in a fed-batch process.

The yeast is concentrated and washed by eg. centrifugation and xylose reductase is liberated into the solution by cell disruption methods such as high pressure homogenization or glass bead milling. After clarification of the homogenate by filtration or centrifugation xylose reductase is further purified by chromatographic methods.

For the production of xylitol *in vitro* a crude homogenate or purified xylose reductase is used in an enzyme reactor together with a cofactor (NAD/NADH or NADP/-NADPH) and a cofactor regenerating enzyme such as glucose dehydrogenase, formate dehydrogenase or any other enzyme having good enough stability, requirements for environmental conditions coping with those of xylose reductase and suitable kinetic properties (Bückmann, 1979; Wandrey *et al.*, 1981; 1982; Kulbe *et al.*, 1989). The enzymes and the cofactor are typically kept in the reactor system by ultrafiltration membranes especially those with a negative charge. The cofactors may also be coimmobilized with the cofactor regenerating enzymes (Reslow *et al.*, 1988). The reaction mixture is pumped through the reactor and the substrates and the products are filtered through the membrane. The products can be separated from the substrates by eg. chromatographic or crystallization methods and the substrates can be recycled to the reaction mixture.

Further, this invention provides a microbiological process for producing xylitol which process comprises:

(a) cultivating a recombinant yeast strain carrying a DNA sequence coding for xylose reductase enzyme in xylose containing medium; and

(b) recovering the xylitol formed in the medium.

In a microbiological xylitol production with a recombinant yeast the *in vivo* regeneration of the cofactor NADPH or NADH must be secured in one way or another. With a yeast construction having only xylose reductase and not xylitol dehydrogenase gene, cofactor regeneration can be achieved by adding a co-carbon-substrate such as glucose, glycerol, ribose or ethanol. With such a system, 95-100 % yield of xylitol from xylose can be obtained. In this system with *S. cerevisiae*, xylitol is not metabolized further and consequently higher yields of xylitol can be obtained than with

natural xylitol producing organisms. When the yeast has also xylitol dehydrogenase gene (see hereunder) cofactor regeneration may happen through a slight flow of xylitol further in the metabolism. This flow can be controlled by relative amounts of expression of the enzymes xylose reductase and xylitol dehydrogenase or by controlling the metabolism of the yeast by oxygen transfer rate or by adding enzyme inhibitors such as iodoacetate to the cultivation medium.

In the preferred embodiment, for the isolation of the *xdh* gene of *P. stipitis* a chromosomal gene bank is first made into *E. coli* in a cosmid p3030 (Penttilä, *et al.*, 1984) or in another yeast vector, and recombinant plasmids are isolated and transformed into yeast. The *xdh* gene can be found by its expression in yeast, which can be detected by an activity plate assay. A cDNA copy for this gene can be isolated similarly by an activity plate assay from a λ gt11 cDNA expression library made in *E. coli*.

An alternative method for the isolation of the *xdh* gene from *P. stipitis* is to purify XDH from a donor yeast by chromatographic methods and determine the N-terminal amino acid sequence thereof. A mixture of oligonucleotides based on the obtained N-terminal amino acid sequence of XDH protein can be designed. This oligonucleotide mixture can then be used in hybridization of a gene bank, or together with an oligo-dT-primer to amplify by PCR reaction *xdh* specific sequences from a mRNA population. The resulting gene or cDNA is cloned into BS+ vector, or a similar vector, and the sequence of the *xdh* gene is then obtained by conventional methods.

It is thus also an object of this invention to provide a specific xylitol dehydrogenase gene.

The *xdh* gene can be expressed in yeast from the chromosomal copy cloned into for instance the yeast cosmid p3030 (Penttilä *et al.*, 1984). To such a yeast carrying the *xdh* gene, the plasmid carrying the *xrd* gene can be transformed.

Also, the full length *xdh* cDNA can be cloned into a suitable expression vector, such as pMA91 or pKB102 (Blomqvist *et al.*, 1991) in between appropriate yeast regulatory regions, preferably using the yeast *PGK1* or *ADH1* promoter and terminator. The

expression cassette built into pKB102 can be released from the resulting plasmid and cloned into an autonomously replicating yeast multicopy vector or into a single copy yeast vector, which carry a suitable marker, eg. *URA3* or *HIS3* for yeast transformation. These resulting plasmids can then be transformed into a suitable host strain or into the strains carrying the gene encoding XR.

The *xdh* gene can also be integrated into the yeast genome in the same manner as described above for the *xrd* gene.

Thus, a further object of this invention is to provide a method for constructing new yeast strains capable of expressing xylose reductase or xylitol dehydrogenase or coexpressing xylose reductase and xylitol dehydrogenase, which method comprises:

(a) isolating the DNA sequences coding for xylose reductase and xylitol dehydrogenase from a suitable donor organism;
(b) constructing a yeast vector carrying either of the said DNA sequences; and
(c) transforming either of the vectors obtained or both of them into a suitable yeast host.

The present invention thus provides a process for coexpressing active xylose reductase and xylitol dehydrogenase in a yeast strain, which process comprises:

(a) isolating the DNA sequences coding for xylose reductase and xylitol dehydrogenase from a suitable donor organism;
(b) constructing yeast vectors each carrying one of said DNA sequences;
(c) transforming the vectors obtained to a suitable host to obtain a recombinant yeast strain;
(d) cultivating said recombinant yeast strain in a xylose containing medium; and
(e) isolating and purifying the products (ethanol, xylitol, acetic acid) formed in the medium.

The recombinant yeast strains according to this invention coexpressing xylose reductase and xylitol dehydrogenase enzymes are potent ethanol producers from xylose by fermenting the xylose containing fraction in for instance lignocellulosic hydrolyzates such as by-products from the forest products industry, eg. spent sulphite liquor, or in raw materials which have been obtained by pretreatment to make the

xylose fraction available for fermentation by treatment at elevated temperatures in the presence or absence of chemicals such as sulphur dioxide and in combination with acid or enzymatic hydrolysis. The consumption of xylose and the formation of products (ethanol, xylitol, acetic acid etc.) are analysed for instance by HPLC (Hahn-Hägerdal *et al.*, 1986; Lindén and Hahn-Hägerdal, 1989a, b).

Example 1:

Purification of xylose reductase from *Pichia stipitis*

P. stipitis was grown in a 1 litre fermentor in xylose containing medium (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1.9 % KH_2PO_4, 0.3 % $(NH_4)_2HPO_4$, 0.1 % $MgSO_4$ and 5 % xylose, pH 5) and harvested in late logarithmic growth phase. 30 g wet weight of cell paste was disrupted using freeze pressing (X-press) and centrifuged 1500 g, 10 min to get a cell free extract.

The crude extract was concentrated 2-fold and applied to a Sephadex* G-200 (Pharmacia, Uppsala, Sweden) column (137 ml). At a flow rate of 6 ml/h the proteins were eluted and fractions (9 ml) containing XR activity were pooled.

The pooled fraction was applied to a DEAE-Sepharose*(Pharmacia) column (37 ml) equilibrated with 0.02M ammonium phosphate buffer pH 6.0 and eluted with a 250 ml gradient of 0-0.5 M NaCl in 0.02 M ammonium phosphate buffer at a flow rate of 12 ml/min. Fractions containing XR activity were pooled (6 ml) and concentrated to 1 ml. 1 ml of the concentrated sample was applied to a 1 ml HPLAC column (cibacron* blue F36-A; Perstorp Biolytica, Lund, Sweden) equilibrated with 0.02 M ammonium phosphate buffer pH 6.0. Elution of XR was performed with a 0-2 M NaCl gradient in ammonium phosphate buffer at a flow rate of 1 ml/min. Fractions containing XR activity were pooled (6 ml) and dialysed over night at 4°C.

Purity and molecular weight was determined by gradient SDS-polyacrylamide gel (T 8.8-21.3 %, C 2.6 %) electrophoresis (Laemmli, 1970) and native gradient polyacrylamide gel (Pharmacia premade gel, 4/30) electrophoresis (Pharmacia vertical electrophoresis system). Low and high molecular weight standards from Pharmacia were used. Staining of gel was performed with 0.1 % Coomassie* Blue R-250 (Sigma) in 25 % methanol and 10 % acetic acid. In SDS-PAGE and in native-PAGE gel the XR fraction after HPLAC appeared as a single band (data not shown). Specific staining of XR with the zymogram technique showed that the single band in the native-PAGE gel was XR (data not shown). Molecular weight estimation with SDS-PAGE (see Fig. 6) and native-PAGE showed a molecular weight of 38000 ± 1000 for the subunits and 76000 ± 1000 for the native protein.

* Trade Marks

The purified enzyme was used to produce polyclonal antibodies and to make a N-terminal amino acid sequence of the enzyme (Marc Bauman at Dept. of Medical Chemistry, Univ. of Helsinki) (SEQ ID NO. 1).

Example 2:

Cloning of the gene coding for XR from *Pichia stipitis*

1 litre of *P. stipitis* culture was grown in xylose containing medium (see example 1) and harvested in late log phase. Harvested cells were converted to sphearoplasts with Zymolyase, suspended into 60 ml of GuSCN solution and RNA was isolated according to Chirgwin *et al.* (1979). RNA was then run through an oligo(dT) cellulose affinity chromatography column. Poly (A+)mRNA was eluted by decreasing the ionic strength of the elution buffer. cDNA was synthesized from mRNA using the cDNA synthesis kit of Amersham and cloned into the λ gt11 vector using the Amersham cDNA cloning kit. After 3-4 h growth, the plaques were replica plated onto nitrocellulose membranes soaked in IPTG, and incubated over night. The membranes were then used for immunoscreening of transformants (Young and Davies, 1983) using rabbit antiserum against XR and goat anti rabbit antibodies coupled with alkaline phosphatase. Positive clones were picked from the plates and the insert DNA was amplified with PCR (Güssow and Clackson, 1989) using vector specific primers. The DNA fragments obtained were used for restriction enzyme analysis and for further cloning after BamHI cleavage into BamHI cleaved BS+ (Stratagene) vector. The longest cDNA clone pJHXR20 was sequenced using the double stranded dideoxy nucleotide method (Zagursky *et al.*, 1986).

Verification of the cloning of the full length cDNA coding for XR was obtained by comparing the N-terminal amino acid sequence of the protein with the sequenced gene (SEQ ID NO. 1, SEQ ID NO. 2).

The chromosomal copy of the *xrd* gene was obtained by PCR reaction of total chromosomal DNA isolated (Cryer *et al.*,1975) from *P. stipitis* CBS-6054 (Prior *et al.*, 1989) using primers corresponding to the 5' (GCGGATCCTCTAGAATGCCTT-

CTATTAAGTTGAACTCTGG) and to the 3' (TTGGATCCTCTAGATTAGAC-GAAGATAGGAATCTTGTCCC) end of the coding region and carrying BamHI and XbaI restriction sites. The PCR product was digested with BamHI and cloned into plasmid pMA91 (Mellor *et al.*, 1983) at the BglII site to obtain plasmid pUA103 (Fig. 1). The DNA sequence of the chromosomal copy was compared to that of the cDNA in plasmid pJHXR20, and was shown to contain no introns.

Example 3:

Purification of XDH from *Pichia stipitis*

Pichia stipitis was grown in xylose containing medium as described (example 1). 30 g wet weight of cell paste was disrupted using freeze pressing (X-press) and centrifuged 1500 g 10 min. The centrifuged cell free extract was concentrated 3 times and 5 ml was gel filtrated at a flow rate of 6 ml/h through a 137 ml Sepharose 6B column equilibrated with 0.05 M ammonium phosphate buffer pH 6, 25 % glycerol, 1 mM DTT, 1 mM EDTA. Fractions containing XDH activity measured according to Smiley and Bolen (1982) were pooled and applied on a 37 ml ion exchange chromatography column (DEAE-sepharose) equilibrated with 0.05 M ammonium phosphate buffer pH 6, 25 % glycerol, 1 mM DTT, 1 mM EDTA. XDH fractions were eluted with a 250 ml salt gradient of 0-0.5 M NaCl at a flow rate of 12 ml/min and pooled. The partially purified enzyme was run in a polyacrylamide gel and blotted onto a PVD membrane. The band corresponding to XDH was cut out and the N-terminal amino acid sequence was determined directly from the membrane.

Example 4:

Cloning of the gene coding for XDH and expression in *S. cerevisiae*

The same λ gt11 cDNA library as obtained and described in Example 2 was plated and replica plated onto nitrocellulose membrane soaked in IPTG and incubated for 3 hours. The membranes were then used for specific XDH activity (zymogram) screening by soaking the membranes in 10 ml of zymogram solution (0.1 M phosphate buffer pH 7.0, 1.5 mM NAD, 0.25 mM nitroblue tetrazolium, 65 µM phenazine

methosulphate, 0.4 M xylitol). Positive clones (Fig. 2) were picked from the plates and the insert DNA of two of the clones was amplified with PCR (Güssow and Clackson, 1989) using vector specific primers (5'-GGTGGCGACGACTCCTG-GAGCCCG, 5'-TTGACACCAGACCAACTGGTAATG). The DNA fragments obtained were of the same size and were used for restriction enzyme analysis to check non-cutting and cutting enzymes and for further cloning after BamHI cleavage into BamHI cleaved pSP72 vector (Promega). The plasmid pJHXDH50 carries the longest cDNA clone (Fig. 3).

To clone the chromosomal copy of the *xdh* gene, chromosomal DNA was isolated (Cryer *et al.*, 1975) from *P. stipitis* strain CBS-6054 (Prior *et al.*, 1989) and cut partially with Sau 3A. Fragments of 35-45 kb in size were purified by fractionating the partially digested DNA in a sucrose gradient (15 %-40 %), and the fragments were ligated to a p3030 yeast cosmid vector (Penttilä *et al.*, 1984), cut with BamHI. The ligated molecules were packaged into λ particles using the *in vitro* packaging kit of Amersham Ltd. (UK) and transfected into *E. coli* HB101 (Maniatis *et al.*, 1982). Recombinant cosmid DNA was isolated from about 15000 pooled gene bank clones obtained and transformed into a *S. cerevisiae* strain S150-2B (*a*, *his3-del1*, *leu2-3*, *leu2-112*, *trp1-289*, *ura3-52*, cir°, Gal°) (Baldari *et al.*, 1987) by selecting for His° transformants on Sc-his plates. The gene bank was replica plated onto nitrocellulose filters, the cells were broken by incubating the filters for 5 min. in chloroform and the activity assay was performed as described above for the λ gt11 library. Several positive clones were obtained (strains H494, H495, H496, H497 and VTT-C-91181), and the strain VTT-C-91181 (Fig. 4) was used for further studies.

XDH activity of the strain VTT-C-91181 was tested according to Smiley and Bolen (1982) from French pressed total cell lysate containing 25 % glycerol. DNA from VTT-C-91181 was isolated and the plasmid pMW22 rescued by transformation of the DNA into *E. coli* DH5α.

The *S. cerevisiae* strain VTT-C-91181 carrying the plasmid pMW22 was deposited according to the Budapest Treaty with the accession No. NCYC 2352 at the National Collection of Yeast Cultures (NCYC), UK, on March 29, 1991.

Example 5:

Expression of XR in *S. cerevisiae*

The gene coding for XR, with the regulatory regions of the *PGK* gene, was released from the plasmid pUA103 (Example 2) with HindIII and cloned at the HindIII site of the single copy vector pRS305 (Sikorski and Hieter, 1989). The resulting plasmid pUA107 (Fig. 1), in which the XR encoding gene was in the right orientation towards the yeast *PGK* promoter, was transformed using the LiCl method (Ito *et al.*, 1983), and selecting for Leu$^+$ transformants, into *S. cerevisiae* strains S150-2B (see Example 4) and GPY55-15Bα (*leu2-3, leu2-112, ura3-52, trp1-289, his4-519, prb1*, cir^0) giving the new recombinant strains *S. cerevisiae* H481 and *S. cerevisiae* H479, respectively. The plasmid pUA103 transformed into the strains S150-2B and GPY-15Bα resulted in strains H477 and H475, respectively.

The gene coding for XR was also integrated into the yeast chromosome into the ribosomal RNA loci. The ribosomal sequences of plasmid pIRL9 were released with EcoRI, blunt-ended and cloned at the blunt-ended XbaI site of BS+ to obtain vector pJHR21. The gene coding for XR, coupled in between the *PGK* promoter and terminator was released from the vector pUA103 as a HindIII fragment, blunt-ended and cloned at the blunt-ended XbaI site in the ribosomal sequences of the plasmid pJHR21. From this resulting plasmid pJHXR22 (Fig. 5), the expression cassette, flanked by ribosomal sequences, was released by cutting in the unique restriction sites of the BS+ polylinker. This fragment was cotransformed into yeast with an autonomously replicating plasmid carrying a marker for transformation. The transformants obtained were screened for the presence of the gene coding for XR by enzyme activity tests as described above and the integration pattern was checked by Southern analysis. The autonomously replicating plasmid was removed from the cells carrying the XR expression cassette by cultivating the cells in non-selective YPD growth medium.

The transformants were grown in minimal selective medium and the expression of XR was analyzed by Western blotting using XR specific antibody and the alkaline phosphatase method of Promega (Fig. 6), and by enzyme activity measurements

(Smiley and Bolen, 1982) from crude extracts of yeast cells broken by French press (Fig. 7).

Example 6:

Purification of XR from recombinant *S. cerevisiae*

A recombinant *S. cerevisiae* strain H477 was cultivated in a 1.5 l fermentor in a Sc-leu medium containing 20 g/l glucose. Growth was followed by turbidity measurements and the cells were collected at late exponential growth phase by centrifugation, washed and resuspended in 0.1 M phosphate buffer pH 7.0 containing 1.5 mM phenyl methyl sulfonyl fluoride, to a yeast concentration of 100 g dry weight/l. Cells were disrupted with 3 passes at 1000 bar through a high pressure homogenizer (French-press). The homogenate was partially clarified by 30 min centrifugation at 15 000 g. XR was purified from the clarified homogenate as described for *P. stipitis* in example 1.

Example 7:

Production of xylitol *in vitro*

A reaction mixture containing 0.33 M xylose, 0.33 M glucose-6-phosphate, 0.67 mM NADPH, 0.1 M phosphate buffer (pH 7.0), 1 nkat/ml XR activity of purified XR (Example 6) or from a diluted crude homogenate of the strain H475, and 1 nkat/ml glucose-6-phosphate dehydrogenase was incubated at 20°C for 5 h. Samples were withdrawn intermittently and analysed for xylitol using a xylitol kit from Boehringer. A constant xylitol production rate exceeding 0.14 g $h^{-1}l^{-1}$ was observed.

Example 8:

Co-expression of XR and XDH

The plasmids pUA103 and pUA107 were transformed into the strain VTT-C-91181 which carries the *xdh* gene on the plasmid pMW22. Transformants were selected, and

also later kept, on Sc-leu-his-plates. The retainment of XDH activity was confirmed by a plate activity assay and the XR activity by enzyme activity measurement as described above. The two clones studied further, carrying the plasmids pUA103 and pMW22, were named H492 and H493.

The full length *xdh* cDNA from the plasmid pJHXDH50 was cloned at the HindIII site in the vector pKB102 (Blomqvist *et al.* 1991) in between the yeast *ADH1* promoter and terminator. The expression cassette was released with BamHI from the resulting plasmid pJHXDH60 (Fig. 8) and cloned into the autonomously replicating yeast vector p3030 at the BamHI site generating the plasmid pJHXDH70. The resulting plasmid was transformed into the strain H477 carrying the gene encoding XR (Example 5) selecting the transformants on Sc-leu-his-plates. The expression of the *xdh* gene in *S. cerevisiae* was tested by enzyme activity measurement (Smiley and Bolen, 1982).

Example 9:

Production of xylitol *in vivo* by recombinant *S. cerevisiae*

The yeast strains H475 and H477 were cultivated in a 1.5 l fermentor in a medium containing 10 g/l yeast extract, 20 g/l bacto-peptone and 20 g/l glucose. Cultivation temperature was 30°C. pH was controlled between 4.5 and 8.0. Agitation speed was 400 rpm and aeration rate 0.5 vvm. When glucose was consumed according to the analysis of the samples from the broth, a feed of a solution containing 1 g glucose and 19 g xylose in 100 ml was started at a rate of 0.09 ml/min. After 83 hours of total cultivation time, xylitol concentration in the broth was 12.5 g/l as analysed by HPLC. Thus over 95 % yield of xylitol from xylose fed to the culture was achieved. By using the control strain carrying the vector pMA91 less than 8 % of the xylose was consumed in an analogous experiment.

Example 10:

Xylose fermentation by recombinant *S. cerevisiae*

S. cerevisiae strains H492 and H493 described in example 9 were cultivated on a rotary shaker in Sc-leu-his medium containing 20 g/l glucose. Rotating speed was 200 rpm. When both the glucose and ethanol formed were consumed, the broths were used as inoculum for fermentation on a rotary shaker in Sc-leu-his medium containing 20 g/l xylose. Rotating speed was 90 rpm. The consumption of xylose and the formation of ethanol and xylitol were followed during fermentation by taking samples and analysing them by HPLC (Hahn-Hägerdal *et al.*, 1986; Linden and Hahn-Hägerdal, 1989a, b).

Example 11:

Fermentation of xylose containing raw materials with recombinant *S. cerevisiae*

S. cerevisiae strains H492 and H493 were cultivated as in example 10 in a fermentation medium, where xylose was replaced by spent sulphite liquor. Xylose was converted to cells, ethanol and xylitol.

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2090122

Deposited microorganism

The following yeast strain was deposited according to the Budapest Treaty at the National Collection of Yeast Cultures (NCYC), AFRC Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich, NR4 7UA, UK

Strain	Deposition number	Deposition date
Saccharomyces cerevisiae VTT-C-91181 carrying the plasmid pMW22	NCYC 2352	March 29, 1991

Sequence Listing

SEQ ID NO. 1

SEQUENCE TYPE: Peptide

SEQUENCE LENGTH: 9 amino acids

TOPOLOGY: linear

ORIGINAL SOURCE ORGANISM: *Pichia stipitis* CBS-6054

IMMEDIATE EXPERIMENTAL SOURCE: protein purification and N-terminal sequencing

FEATURES: from 1 to 9 amino acids of the mature protein

PROPERTIES: N-terminal peptide of xylose reductase (NADPH/NADH) enzyme

Pro Xaa Ile Lys Leu Asn Ser Gly Tyr

SEQ ID NO. 2

SEQUENCE TYPE: Nucleotide with corresponding protein

SEQUENCE LENGTH: 954 base pairs

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA, genomic DNA

ORIGINAL SOURCE ORGANISM: *Pichia stipitis* CBS-6054

IMMEDIATE EXPERIMENTAL SOURCE: cDNA library, PCR product from genomic DNA

FEATURES: from 1 to 954 bp mature protein

PROPERTIES: xylose reductase (NADPH/NADH) activity of the product

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ATG CCT TCT ATT AAG TTG AAC TCT GGT TAC GAC ATG CCA GCC GTC      45
Met Pro Ser Ile Lys Leu Asn Ser Gly Tyr Asp Met Pro Ala Val
 1               5                  10                  15

GGT TTC GGC TGT TGG AAA GTC GAC GTC GAC ACC TGT TCT GAA CAG      90
Gly Phe Gly Cys Trp Lys Val Asp Val Asp Thr Cys Ser Glu Gln
            20                  25                  30

ATC TAC CGT GCT ATC AAG ACC GGT TAC AGA TTG TTC GAC GGT GCC     135
Ile Tyr Arg Ala Ile Lys Thr Gly Tyr Arg Leu Phe Asp Gly Ala
        35                  40                  45

GAA GAT TAC GCC AAC GAA AAG TTA GTT GGT GCC GGT GTC AAG AAG     180
Glu Asp Tyr Ala Asn Glu Lys Leu Val Gly Ala Gly Val Lys Lys
    50                  55                  60

GCC ATT GAC GAA GGT ATC GTC AAG CGT GAA GAC TTG TTC CTT ACC     225
Ala Ile Asp Glu Gly Ile Val Lys Arg Glu Asp Leu Phe Leu Thr
65                  70                  75

TCC AAG TTG TGG AAC AAC TAC CAC CAC CCA GAC AAC GTC GAA AAG     270
Ser Lys Leu Trp Asn Asn Tyr His His Pro Asp Asn Val Glu Lys
            80                  85                  90

GCC TTG AAC AGA ACC CTT TCT GAC TTG CAA GTT GAC TAC GTT GAC     315
Ala Leu Asn Arg Thr Leu Ser Asp Leu Gln Val Asp Tyr Val Asp
        95                 100                 105

TTG TTC TTG ATC CAC TTC CCA GTC ACC TTC AAG TTC GTT CCA TTA     360
Leu Phe Leu Ile His Phe Pro Val Thr Phe Lys Phe Val Pro Leu
    110                 115                 120

GAA GAA AAG TAC CCA CCA GGA TTC TAC TGT GGT AAG GGT GAC AAC     405
Glu Glu Lys Tyr Pro Pro Gly Phe Tyr Cys Gly Lys Gly Asp Asn
125                 130                 135

TTC GAC TAC GAA GAT GTT CCA ATT TTA GAG ACC TGG AAG GCT CTT     450
Phe Asp Tyr Glu Asp Val Pro Ile Leu Glu Thr Trp Lys Ala Leu
            140                 145                 150

GAA AAG TTG GTC AAG GCC GGT AAG ATC AGA TCT ATC GGT GTT TCT     495
Glu Lys Leu Val Lys Ala Gly Lys Ile Arg Ser Ile Gly Val Ser
        155                 160                 165

AAC TTC CCA GGT GCT TTG CTC TTG GAC TTG TTG AGA GGT GCT ACC     540
Asn Phe Pro Gly Ala Leu Leu Leu Asp Leu Leu Arg Gly Ala Thr
    170                 175                 180

ATC AAG CCA TCT GTC TTG CAA GTT GAA CAC CAC CCA TAC TTG CAA     585
Ile Lys Pro Ser Val Leu Gln Val Glu His His Pro Tyr Leu Gln
185                 190                 195

CAA CCA AGA TTG ATC GAA TTC GCT CAA TCC CGT GGT ATT GCT GTC     630
Gln Pro Arg Leu Ile Glu Phe Ala Gln Ser Arg Gly Ile Ala Val
            200                 205                 210

ACC GCT TAC TCT TCG TTC GGT CCT CAA TCT TTC GTT GAA TTG AAC     675
Thr Ala Tyr Ser Ser Phe Gly Pro Gln Ser Phe Val Glu Leu Asn
        215                 220                 225

CAA GGT AGA GCT TTG AAC ACT TCT CCA TTG TTC GAG AAC GAA ACT     720
Gln Gly Arg Ala Leu Asn Thr Ser Pro Leu Phe Glu Asn Glu Thr
    230                 235                 240

ATC AAG GCT ATC GCT GCT AAG CAC GGT AAG TCT CCA GCT CAA GTC     765
Ile Lys Ala Ile Ala Ala Lys His Gly Lys Ser Pro Ala Gln Val
245                 250                 255

TTG TTG AGA TGG TCT TCC CAA AGA GGC ATT GCC ATC ATT CCA AAG     810
Leu Leu Arg Trp Ser Ser Gln Arg Gly Ile Ala Ile Ile Pro Lys
            260                 265                 270

TCC AAC ACT GTC CCA AGA TTG TTG GAA AAC AAG GAC GTC AAC AGC     855
Ser Asn Thr Val Pro Arg Leu Leu Glu Asn Lys Asp Val Asn Ser
        275                 280                 285

TTC GAC TTG GAC GAA CAA GAT TTC GCT GAC ATT GCC AAG TTG GAC     900
Phe Asp Leu Asp Glu Gln Asp Phe Ala Asp Ile Ala Lys Leu Asp
    290                 295                 300

ATC AAC TTG AGA TTC AAC GAC CCA TGG GAC TGG GAC AAG ATT CCT     945
Ile Asn Leu Arg Phe Asn Asp Pro Trp Asp Trp Asp Lys Ile Pro
305                 310                 315

ATC TTC GTC                                                    954
Ile Phe Val
```

Claims:

1. A process for producing xylitol, which process comprises

(a) cultivating in a xylose containing medium a yeast strain transformed with a DNA sequence encoding a xylose reductase enzyme, which DNA sequence when transformed into said yeast strain confers to said yeast strain the ability of reducing xylose to xylitol, and

(b) recovering xylitol formed from the medium.

2. The process of claim 1, wherein said DNA sequence encodes a polypeptide comprising essentially the amino acid sequence of SEQ. ID. NO. 2, or a fragment thereof wherein said polypeptide or fragment displays xylose reductase activity.

3. The process of claim 1 or 2, wherein said transformed yeast strain belongs to the species *Saccharomyces cerevisiae, Kluyveromyces* spp., *Schizosaccharomyces pombe* or *Pichia* spp.

4. The process of any one of claims 1 to 3, wherein said transformed yeast strain belongs to the species *Saccharomyces cerevisiae.*

5. The process of any one of claims 1 to 4, wherein said transformed yeast strain is *Saccharomyces cerevisiae* H475 or H477, obtainable by transforming *Saccharomyces cerevisiae* GPY55-15B*a* and S150-2B, respectively, with plasmid pUA103 as shown in Figure 1.

6. The process of claim 1, wherein at least one additional carbon source is present in the medium to enhance regeneration of a cofactor required for xylose reductase activity.

7. The process of claim 6, wherein said additional carbon source is ethanol, glucose or glycerol.

8. The process of claim 1, wherein said transformed yeast strain is further transformed with a second DNA sequence encoding a xylitol dehydrogenase enzyme, said xylitol dehydrogenase enzyme making possible the regeneration of a cofactor required for xylose reductase activity.

9. The process of claim 8, wherein said transformed yeast strain belongs to the species *Saccharomyces cerevisiae*, *Kluyveromyces* spp., *Schizosaccharomyces pombe* or *Pichia* spp.

10. The process of claim 9 wherein said transformed yeast strain is *Saccharomyces cerevisiae* VTT-C-91181 carrying the plasmid pMW22 (NCYC No. 2352) additionally transformed with plasmid pUA103 as shown in Figure 1.

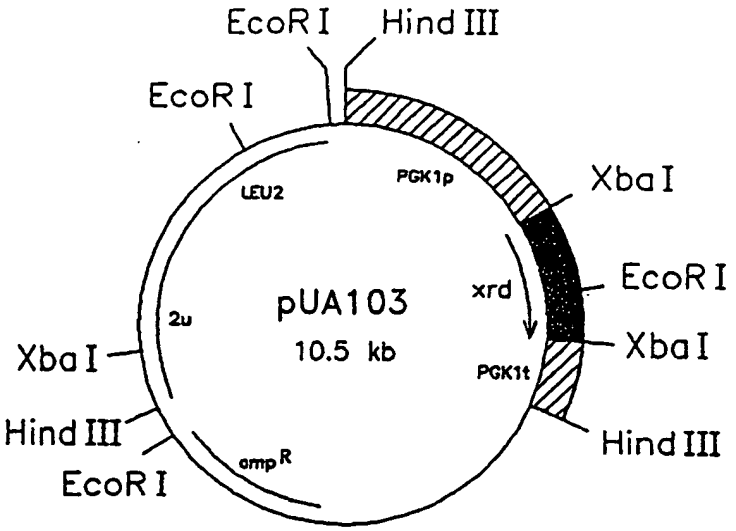
11. An enzymatic process for producing xylitol, which process comprises

(a) cultivating a recombinant yeast strain transformed with a DNA sequence encoding a xylose reductase enzyme under conditions permitting expression of said xylose reductase,

(b) recovering said xylose reductase enzyme,

(c) using said xylose reductase in an enzyme reactor with a cofactor regenerating system, and

(d) isolating and purifying the xylitol produced.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EcoR I
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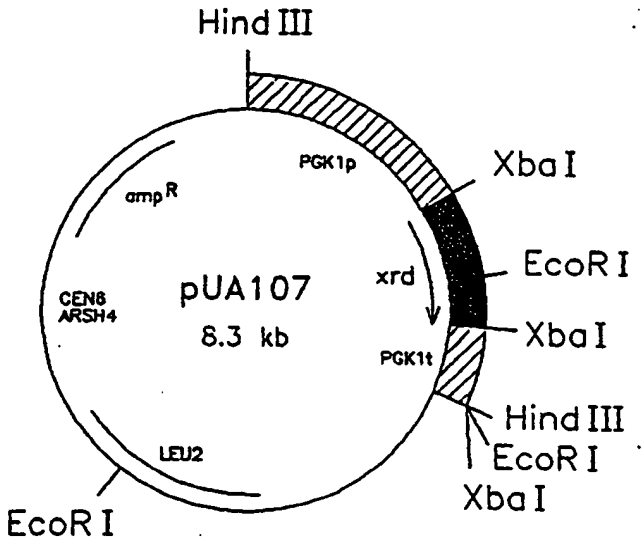
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EcoR I

Fig. 1

2090122

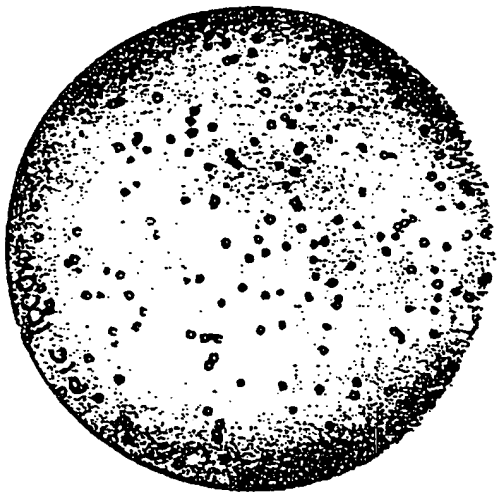
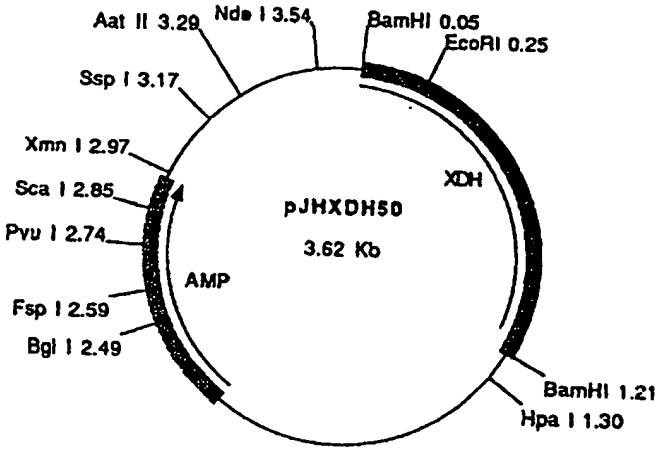


Fig. 2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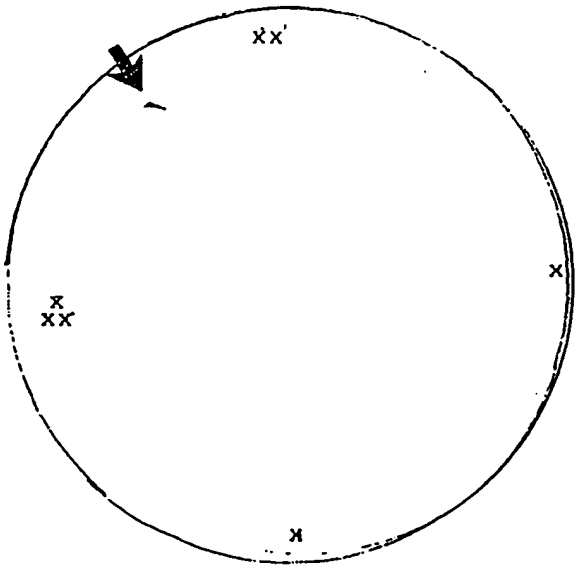
Nde I 3.54
BamHI 0.05
EcoRI 0.25
Ssp I 3.17
Xmn I 2.97
Sca I 2.85
Pvu I 2.74
Fsp I 2.59
Bgl I 2.49
XDH
pJHXDH50
3.62 Kb
AMP
BamHI 1.21
Hpa I 1.30

Fig. 3

A



B

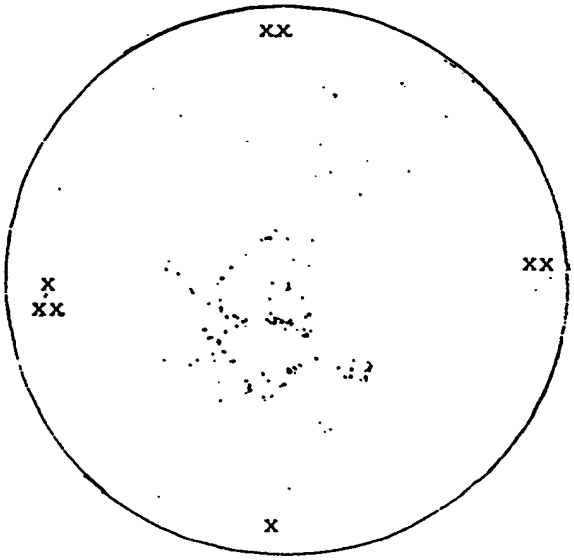
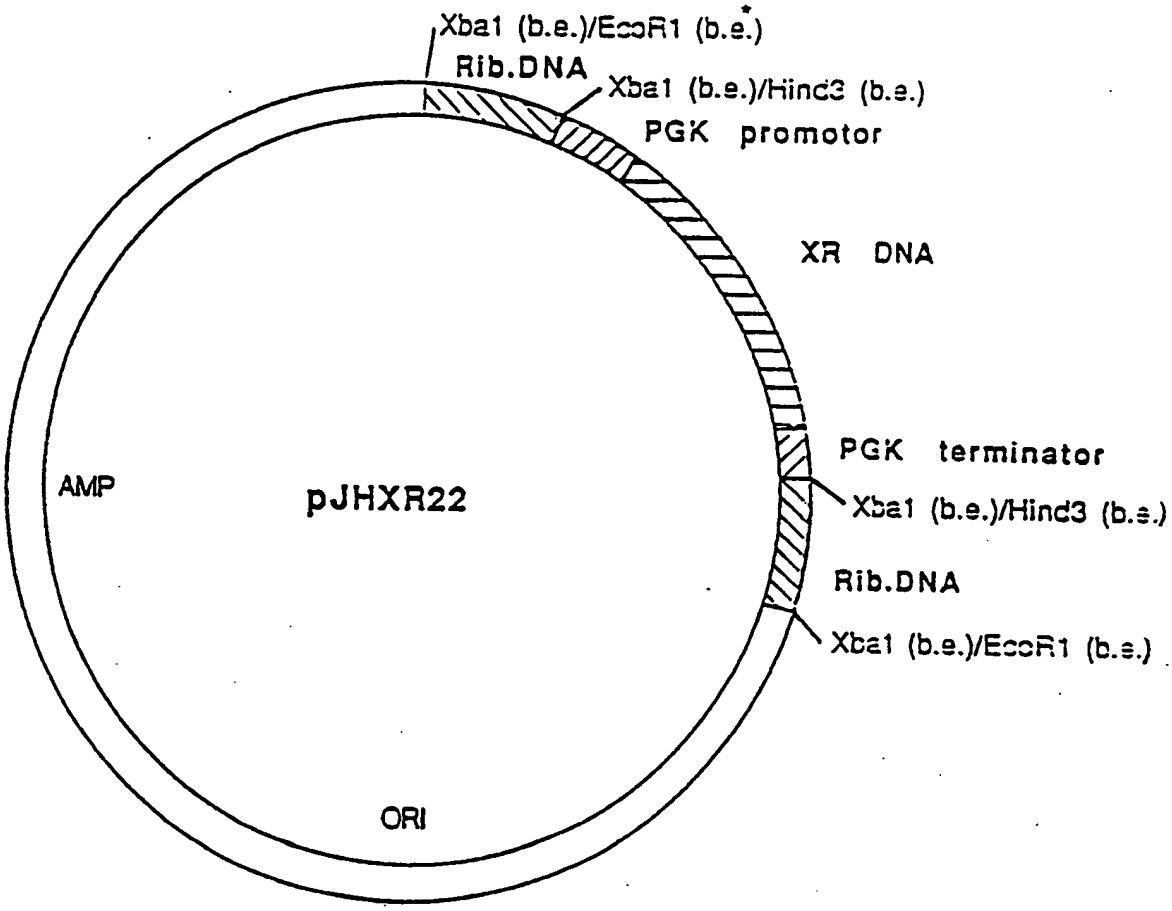


Fig. 4



*) b.e. = blunt ended

Fig. 5

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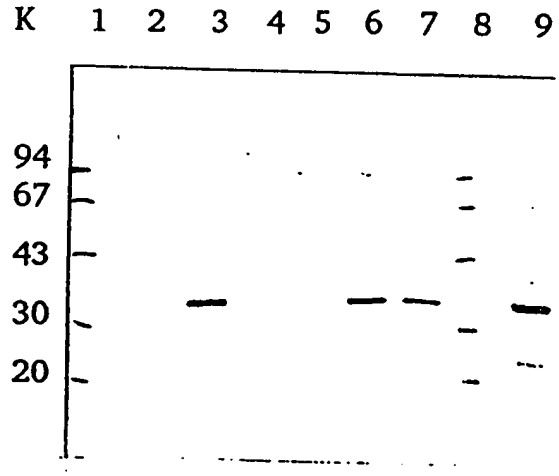
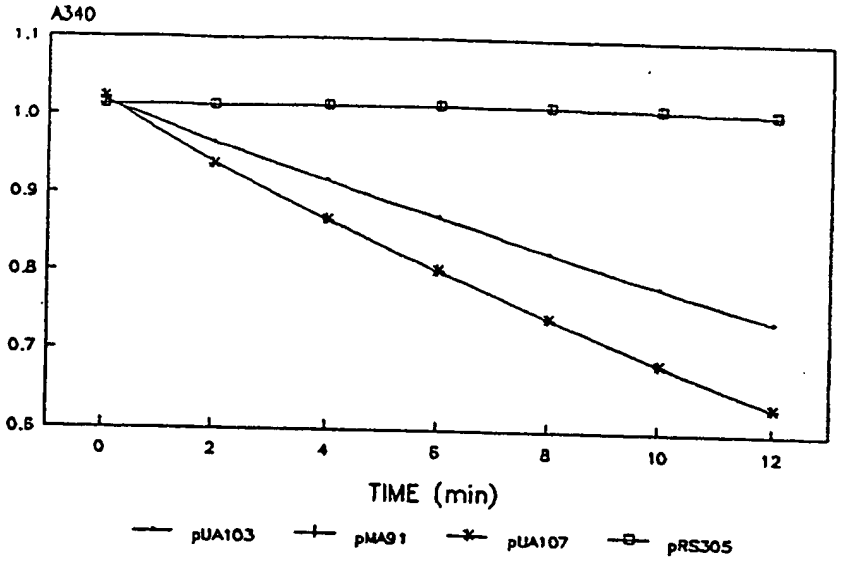


Fig. 6

2090122

XR activity with NADPH



XR activity with NADH

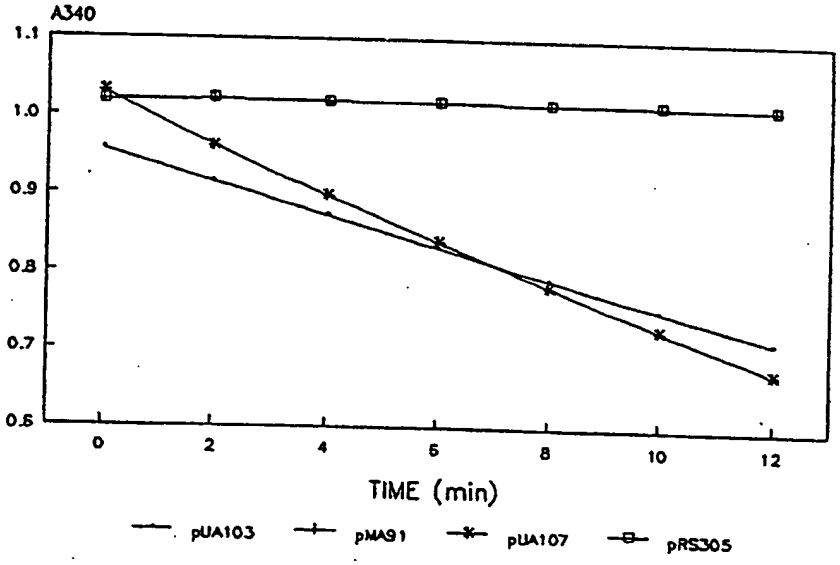


Fig. 7

2090122

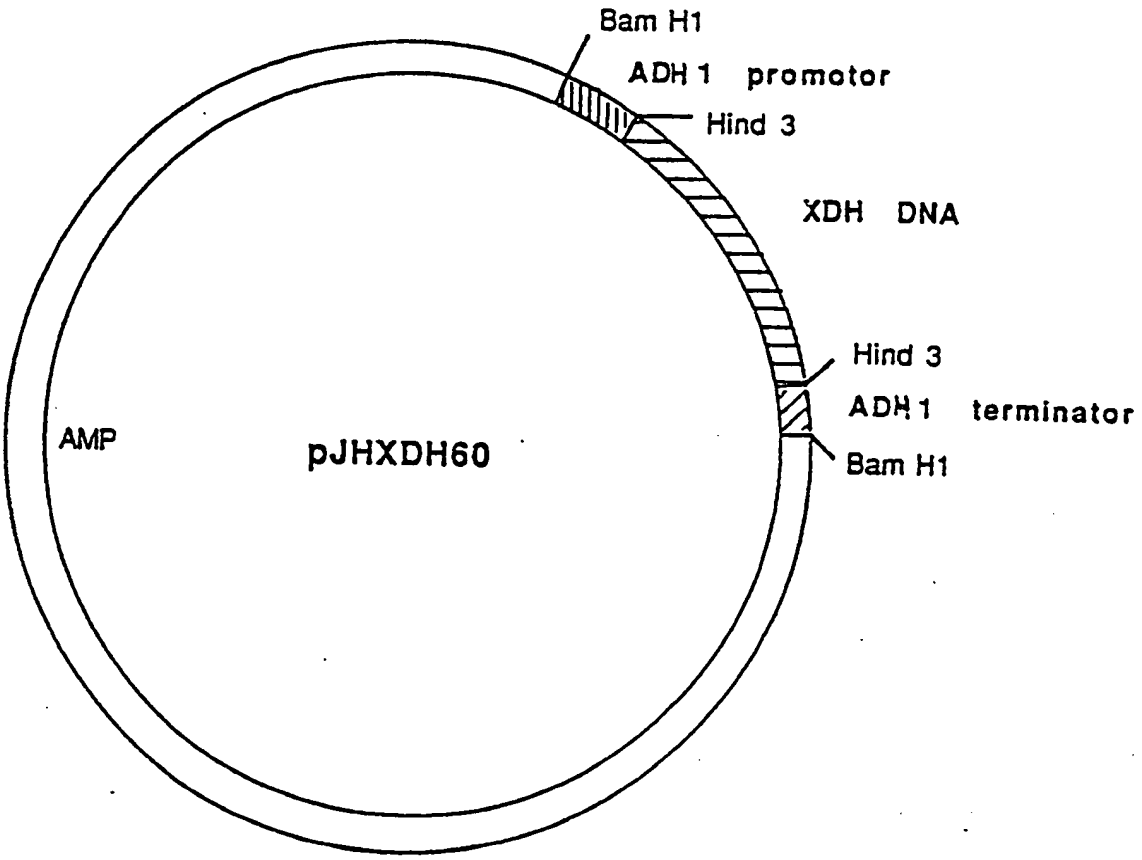


Fig. 8

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FULL TEXT OF CASES (USPQ2D)
All Other Cases

In re Vaeck (CA FC) 20 USPQ2d 1438 In re Vaeck

U.S. Court of Appeals Federal Circuit
20 USPQ2d 1438

Decided October 21, 1991
No. 91-1120

Headnotes

PATENTS

1. Patentability/Validity - Obviousness - Combining references (§ 115.0905)

Rejection of claimed subject matter as obvious under 35 USC 103 in view of combination of prior art references requires consideration of whether prior art would have suggested to those of ordinary skill in art that they should make claimed composition or device, or carry out claimed process, and whether prior art would also have revealed that such person would have reasonable expectation of success; both suggestion and reasonable expectation of success must be founded in prior art, not in applicant's disclosure.

2. Patentability/Validity - Obviousness - Relevant prior art - Particular inventions (§ 115.0903.03)

Patent and Trademark Office has failed to establish prima facie obviousness of claims for use of genetic engineering techniques for producing proteins that are toxic to insects such as larvae of mosquitos and black flies, since prior art does not disclose or suggest expression in cyanobacteria of chimeric gene encoding insecticidally active protein, or convey to those of ordinary skill reasonable expectation of success in doing so; expression of antibiotic resistance-conferring genes in cyanobacteria, without more, does not render obvious expression of unrelated genes in cyanobacteria for unrelated purposes.

3. Patentability/Validity - Specification - Enablement (§ 115.1105)

JUDICIAL PRACTICE AND PROCEDURE

Procedure - Judicial review - Standard of review - Patents (§ 410.4607.09)

Specification must, in order to be enabling as required by 35 USC 112, first paragraph, teach person skilled in art to make and use invention without "undue experimentation," which does not preclude some experimentation; enablement is question of law which is reviewed independently on appeal, although such determination is based upon underlying factual findings which are reviewed for clear error.

PATENTS

4. Patentability/Validity - Specification - Enablement (§ 115.1105)

Patent and Trademark Office did not err in rejecting, as non-enabling pursuant to 35 USC 112, first paragraph, claims for use of genetic engineering techniques for producing proteins that are toxic to insects such as larvae of mosquitos and black flies, in view of relatively incomplete understanding of biology of cyanobacteria as of applicants' filing date, as well as limited disclosure by applicants of particular cyanobacterial genera operative in claimed invention, since there is no reasonable correlation between narrow disclosure in applicants' specification and broad scope of protection sought in claims encompassing gene expression in any and all cyanobacteria.

Case History and Disposition:

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Application for patent, serial no. 07/021,405, filed March 4, 1987, by Mark A. Vaeck, Wipa Chungjatupornchai, and Lee McIntosh (hybrid genes incorporating a DNA fragment containing a gene coding for an insecticidal protein, plasmids, transformed cyanobacteria expressing such protein and method for use as a biocontrol agent). From decision rejecting claims 1-48 and 50-52 as unpatentable under 35 USC 103, and rejecting claims 1-48 and 50-51 for lack of enablement, applicants appeal. Affirmed and part and reversed in part; Mayer, J., dissents with opinion.

Attorneys:

Ian C. McLeod, Okemos, Mich., for appellant.

Teddy S. Gron, associate solicitor (Fred E. McKelvey, solicitor and Richard E. Schafer, associate solicitor, with him on brief), for appellee.

Judge:

Before Rich, Archer, and Mayer, circuit judges.

Opinion Text

Opinion By:

Rich, J.

This appeal is from the September 12, 1990 decision of the Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), affirming the examiner's rejection of claims 1-48 and 50-52 of application Serial No. 07/021,405, filed March 4, 1987, titled "Hybrid Genes Incorporating a DNA Fragment Containing a Gene Coding for an Insecticidal Protein, Plasmids, Transformed Cyanobacteria Expressing Such Protein and Method for Use as a Biocontrol Agent" as unpatentable under 35 USC 103, as well as the rejection of claims 1-48 and 50-51 under 35 USC 112, first paragraph, for lack of enablement. We reverse the § 103 rejection. The § 112 rejection is affirmed in part and reversed in part.

BACKGROUND

A. *The Invention*

The claimed invention is directed to the use of genetic engineering techniques 1 for production of proteins that are toxic to insects such as larvae of mosquitos and black flies. These swamp-dwelling pests are the source of numerous human health problems, including malaria. It is known that certain species of the naturally-occurring *Bacillus* genus of bacteria produce proteins ("endotoxins") that are toxic to these insects. Prior art methods of combatting the insects involved spreading or spraying crystalline spores of the insecticidal *Bacillus* proteins over swamps. The spores were environmentally unstable, however, and would often sink to the bottom of a swamp before being consumed, thus rendering this method prohibitively expensive. Hence the need for a lower-cost method of producing the insecticidal *Bacillus* proteins in high volume, with application in a more stable vehicle. As described by appellants, the claimed subject matter meets this need by providing for the production of the insecticidal *Bacillus* proteins within host cyanobacteria. Although both cyanobacteria and bacteria are members of the procaryote 2 kingdom, the

cyanobacteria (which in the past have been referred to as "blue-green algae") are unique among procaryotes in that the cyanobacteria are capable of oxygenic photosynthesis. The cyanobacteria grow on top of swamps where they are consumed by mosquitos and black flies. Thus, when *Bacillus* proteins are produced within transformed 3 cyanobacterial hosts according to the claimed invention, the presence of the insecticide in the food of the targeted insects advantageously guarantees direct uptake by the insects.

More particularly, the subject matter of the application on appeal includes a chimeric (i.e., hybrid) gene comprising (1) a gene derived from a bacterium of the *Bacillus* genus whose product is an insecticidal protein, united with (2) a DNA promoter effective for expressing 4 the *Bacillus* gene in a host cyanobacterium, so as to produce the desired insecticidal protein.
The claims on appeal are 1-48 and 50-52, all claims remaining in the application. Claim 1 reads:
1. A chimeric gene capable of being expressed in Cyanobacteria cells comprising:
(a) a DNA fragment comprising a promoter region which is effective for expression of a DNA fragment in a Cyanobacterium; and
(b) at least one DNA fragment coding for an insecticidally active protein produced by a Bacillus strain, or coding for an insecticidally active truncated form of the above protein or coding for a protein having substantial sequence homology to the active protein,
the DNA fragments being linked so that the gene is expressed.
Claims 2-15, which depend from claim 1, recite preferred *Bacillus* species, promoters, and selectable markers. 5 Independent claim 16 and claims 17-31 which depend therefrom are directed to a hybrid plasmid vector which includes the chimeric gene of claim 1. Claim 32 recites a bacterial strain. Independent claim 33 and claims 34-48 which depend therefrom recite a cyanobacterium which expresses the chimeric gene of claim 1. Claims 50-51 recite an insecticidal composition. Claim 52 recites a particular plasmid that appellants have deposited.

B. *Appellants' Disclosure*

In addition to describing the claimed invention in generic terms, appellants' specification discloses two particular species of *Bacillus* (*B. thuringiensis, B. sphaericus*) as sources of insecticidal protein; and nine genera of cyanobacteria (*Synechocystis, Anacystis, Synechococcus, Agmenellum, Aphanocapsa, Gloecapsa, Nostoc, Anabaena* and *Ffremyllia*) as useful hosts.
The working examples relevant to the claims on appeal detail the transformation of a single strain of cyanobacteria, i.e., *Synechocystis* 6803. In one example, *Synechocystis* 6803 cells are transformed with a plasmid comprising (1) a gene encoding a particular insecticidal protein ("B.t. 8") from *Bacillus thuringiensis* var. *israelensis,* linked to (2) a particular promoter, the P_L promoter from the bacteriophage Lambda (a virus of *E. coli*). In another example, a different promoter, i.e., the *Synechocystis* 6803 promoter for the rubisco operon, is utilized instead of the Lambda P_L promoter.

C. *The Prior Art*

A total of eleven prior art references were cited and applied, in various combinations, against the claims on appeal. The focus of Dzelzkalns, 6 the primary reference cited against all of the rejected claims, is to determine whether chloroplast promoter sequences can function in cyanobacteria. To that end Dzelzkalns discloses the expression in cyanobacteria of a chimeric gene comprising a chloroplast promoter sequence fused to a gene encoding the enzyme chloramphenicol acetyl transferase (CAT). 7 Importantly, Dzelzkalns teaches the use of the CAT gene as a "marker" gene; this use of antibiotic resistance-conferring genes for selection purposes is a common technique in genetic engineering.

Sekar I, 8 Sekar II, 9 and Ganesan 10 collectively disclose expression of genes encoding certain *Bacillus* insecticidal proteins in the bacterial hosts *B. megaterium, B. subtilis* and *E. coli.*
Friedberg 11 discloses the transformation of the cyanobacterium *Anacystis nidulans* R2 by a plasmid vector comprising the $O_L P_L$ operator-promoter region and a temperature-sensitive repressor gene of the bacteriophage Lambda. While the cyanobacteria are attractive organisms for the cloning of genes involved in photosynthesis,

Friedberg states, problems may still be encountered such as suboptimal expression of the cloned gene, detrimental effects on cell growth of overexpressed, highly hydrophobic proteins, and rapid turnover of some gene products. To address these problems, Friedberg teaches the use of the disclosed Lambda regulatory signals in plasmid vehicles which, it states, have "considerable potential for use as vectors the expression of which can be controlled in *Anacystis* ...."

Miller 12 compares the initiation specificities *in vitro* of DNA-dependent RNA polymerases 13 purified from two different species of cyanobacteria (*Fremyella diplosiphon* and *Anacystis nidulans*), as well as from *E. coli.*

Nierzwicki-Bauer 14 identifies in the cyanobacterium *Anabaena* 7120 the start site for transcription of the gene encoding *rbc* L, the large subunit of the enzyme ribulose-1, 5-bisphosphate carboxylase. It reports that the nucleotide sequence 14-8 base pairs preceding the transcription start site "resembles a good *Escherichia coli* promoter," but that the sequence 35 base pairs before the start site does not.

Chauvat 15 discloses host-vector systems for gene cloning in the cyanobacterium *Synechocystis* 6803, in which the antibiotic resistance-conferring *neo* gene is utilized as a selectable marker.

Reiss 16 studies expression in *E. coli* of various proteins formed by fusion of certain foreign DNA sequences with the *neo* gene.

Kolowsky 17 discloses chimeric plasmids designed for transformation of the cyanobacterium *Synechococcus* R2, comprising an antibiotic-resistant gene linked to chromosomal DNA from the *Synechococcus* cyanobacterium.

Barnes, United States Patent No. 4,695,455, is directed to the treatment with stabilizing chemical reagents of pesticides produced by expression of heterologous genes (such as those encoding *Bacillus* proteins) in host microbial cells such as *Pseudomonas* bacteria. The host cells are killed by this treatment, but the resulting pesticidal compositions exhibit prolonged toxic activity when exposed to the environment of target pests.

D. *The Grounds of Rejection*

1. The § 103 Rejections

Claims 1-6, 16-21, 33-38, 47-48 and 52 (which include all independent claims in the application) were rejected as unpatentable under 35 USC 103 based upon Dzelzkalns in view of Sekar I or Sekar II and Ganesan. The examiner stated that Dzelzkalns discloses a chimeric gene capable of being highly expressed in a cyanobacterium, said gene comprising a promoter region effective for expression in a cyanobacterium operably linked to a structural gene encoding CAT. The examiner acknowledged that the chimeric gene and transformed host of Dzelzkalns differ from the claimed invention in that the former's structural gene encodes CAT rather than insecticidally active protein. However, the examiner pointed out, Sekar I, Sekar II, and Ganesan teach genes encoding insecticidally active proteins produced by *Bacillus,* and the advantages of expressing such genes in heterologous 18 hosts to obtain larger quantities of the protein. The examiner contended that it would have been obvious to one of ordinary skill in the art to substitute the *Bacillus* genes taught by Sekar I, Sekar II, and Ganesan for the CAT gene in the vectors of Dzelzkalns in order to obtain high level expression of the *Bacillus* genes in the transformed cyanobacteria. The examiner further contended that it would have been obvious to use cyanobacteria as heterologous hosts for expression of the claimed genes due to the ability of cyanobacteria to serve as transformed hosts for the

expression of heterologous genes. In the absence of evidence to the contrary, the examiner contended, the invention as a whole was prima facie obvious.

Additional rejections were entered against various groups of dependent claims which we need not address here. All additional rejections were made in view of Dzelzkalns in combination with Sekar I, Sekar II, and Ganesan, and further in view of other references discussed in Part C above.

The Board affirmed the § 103 rejections, basically adopting the examiner's Answer as its opinion while adding a few comments. The legal conclusion of obviousness does not require absolute certainty, the Board added, but only a reasonable expectation of success, citing *In re O'Farrell,* 853 F.2d 894, 7 USPQ2d 1673 (Fed. Cir. 1988). In view of the disclosures of the prior art, the Board concluded, one of ordinary skill in the art would have been motivated by a reasonable expectation of success to make the substitution suggested by the examiner.

2. The § 112 Rejection

The examiner also rejected claims 1-48 and 50-51 under 35 USC 112, first paragraph, on the ground that the disclosure was enabling only for claims limited in accordance with the specification as filed. Citing *Manual of Patent Examining Procedure* (MPEP) provisions 706.03(n) 19 and (z) 20 as support, the examiner took the position that undue experimentation would be required of the art worker to practice the claimed invention, in view of the unpredictability in the art, the breadth of the claims, the limited number of working examples and the limited guidance provided in the specification. With respect to unpredictability, the examiner stated that
he cyanobacteria comprise a large and diverse group of photosynthetic bacteria including large numbers of species in some 150 different genera including *Synechocystis, Anacystis, Synechococcus, Agmenellum, Nostoc, Anabaena,* etc. The molecular biology of these organisms has only recently become the subject of intensive investigation and this work is limited to a few genera. Therefore the level of unpredictability regarding heterologous gene expression in this large, diverse and relatively poorly studied group of procaryotes is high....
The Board affirmed, noting that "the limited guidance in the specification, considered in light of the relatively high degree of unpredictability in this particular art, would not have enabled one having ordinary skill in the art to practice the broad scope of the claimed invention without undue experimentation. *In re Fisher,* 427 F.2d 833, 166 USPQ 18 (CCPA 1970)."

OPINION

A. *Obviousness*

We first address whether the PTO erred in rejecting the claims on appeal as prima facie obvious within the meaning of 35 USC 103. Obviousness is a legal question which this court independently reviews, though based upon underlying factual findings which we review under the clearly erroneous standard. *In re Woodruff,* 919 F.2d 1575, 1577, 16 USPQ2d 1934, 1935 (Fed. Cir. 1990).
[1] Where claimed subject matter has been rejected as obvious in view of a combination of prior art references, a proper analysis under § 103 requires, *inter alia,* consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. *See In re Dow Chemical Co.,* 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in the applicant's disclosure. *Id.*

[2] We agree with appellants that the PTO has not established the prima facie obviousness of the claimed subject matter. The prior art simply does not disclose or suggest the expression in cyanobacteria of a chimeric gene encoding an insecticidally active protein, or convey to those of ordinary skill a reasonable expectation of success in doing so. More particularly, there is no suggestion in Dzelzkalns, the primary reference cited against all claims, of substituting in the disclosed plasmid a structural gene encoding *Bacillus* insecticidal proteins for the CAT gene utilized for selection purposes. The expression of antibiotic resistance-conferring genes in cyanobacteria, without more, does not render obvious the expression of unrelated genes in cyanobacteria for unrelated purposes.

The PTO argues that the substitution of insecticidal *Bacillus* genes for CAT marker genes in cyanobacteria is suggested by the secondary references Sekar I, Sekar II, and Ganesan, which collectively disclose expression of genes encoding *Bacillus* insecticidal proteins in two species of host *Bacillus* bacteria (*B. megaterium* and *B. subtilis*) as well as in the bacterium *E. coli.* While these references disclose expression of *Bacillus* genes encoding insecticidal proteins in certain transformed *bacterial* hosts, nowhere do these references disclose or suggest expression of such genes in transformed *cyanobacterial* hosts.

To remedy this deficiency, the PTO emphasizes similarity between bacteria and cyanobacteria, namely, that these are both procaryotic organisms, and argues that this fact would suggest to those of ordinary skill the use of cyanobacteria as hosts for expression of the claimed chimeric genes. While it is true that bacteria and cyanobacteria are now both classified as procaryotes, that fact alone is not sufficient to motivate the art worker as the PTO contends. As the PTO concedes, cyanobacteria and bacteria are not identical; they are classified as two separate divisions of the kingdom Procaryotae. 21 Moreover, it is only in recent years that the biology of cyanobacteria has been clarified, as evidenced by references in the prior art to "blue-green algae." Such evidence of recent uncertainty regarding the biology of cyanobacteria tends to rebut, rather than support, the PTO's position that one would consider the cyanobacteria effectively interchangeable with bacteria as hosts for expression of the claimed gene.

At oral argument the PTO referred to additional secondary references, not cited against any independent claim (i.e., Friedberg, Miller, and Nierzwicki-Bauer), which it contended disclose certain amino acid sequence homology between bacteria and cyanobacteria. The PTO argued that such homology is a further suggestion to one of ordinary skill to attempt the claimed invention. We disagree. As with the Dzelzkalns, Sekar I, Sekar II, and Ganesan references discussed above, none of these additional references disclose or suggest that cyanobacteria could serve as hosts for expression of genes encoding *Bacillus* insecticidal proteins. In fact, these additional references suggest as much about *differences* between cyanobacteria and bacteria as they do about similarities. For example, Nierzwicki-Bauer reports that a certain nucleotide sequence (i.e., the -10 consensus sequence) in a particular cyanobacterium resembles an *E. coli* promoter, but that another nearby nucleotide sequence (the -35 region) does not. While Miller speaks of certain promoters of the bacteriophage Lambda that are recognized by both cyanobacterial and *E. coli* RNA polymerases, it also discloses that these promoters exhibited differing strengths when exposed to the different polymerases. Differing sensitivities of the respective polymerases to an inhibitor are also disclosed, suggesting differences in the structures of the initiation complexes.

The PTO asks us to agree that the prior art would lead those of ordinary skill to conclude that cyanobacteria are attractive hosts for expression of any and all heterologous genes. Again, we can not. The relevant prior art does indicate that cyanobacteria are attractive hosts for expression of both native and heterologous *genes involved in photosynthesis* (not surprisingly, for the capability of undergoing oxygenic photosynthesis is what makes the cyanobacteria unique among procaryotes). However, these references do not suggest that cyanobacteria would be equally attractive hosts for expression of *unrelated* heterologous genes, such as the claimed genes encoding *Bacillus* insecticidal proteins.

In *O'Farrell,* this court affirmed an obviousness rejection of a claim to a method for

producing a "predetermined protein in a stable form" in a transformed bacterial host. 853 F.2d at 895, 7 USPQ2d at 1674. The cited references included a prior art publication (the Polisky reference) whose three authors included two of the three coinventor-appellants. The main difference between the prior art and the claim at issue was that in Polisky, the heterologous gene was a gene for ribosomal RNA, while the claimed invention substituted a gene coding for a predetermined protein. *Id.* at 901, 7 USPQ2d at 1679. Although, as the appellants therein pointed out, the ribosomal RNA gene is not normally translated into protein, Polisky mentioned preliminary evidence that the transcript of the ribosomal RNA gene was translated into protein, and further predicted that if a gene coding for

a protein were to be substituted, extensive translation might result. *Id.* We thus affirmed, explaining that the prior art explicitly suggested the substitution that is the difference between the claimed invention and the prior art, and presented preliminary evidence suggesting that the [claimed] method could be used to make proteins.
....
... Polisky contained detailed enabling methodology for practicing the claimed invention, a suggestion to modify the prior art to practice the claimed invention, and evidence suggesting that it would be successful.
Id. at 901-02, 7 USPQ2d at 1679-80.
In contrast with the situation in *O'Farrell,* the prior art in this case offers no suggestion, explicit or implicit, of the substitution that is the difference between the claimed invention and the prior art. Moreover, the "reasonable expectation of success" that was present in *O'Farrell* is not present here. Accordingly, we reverse the § 103 rejections.

B. *Enablement*

[3] The first paragraph of 35 USC 112 requires, *inter alia,* that the specification of a patent enable any person skilled in the art to which it pertains to make and use the claimed invention. Although the statute does not say so, enablement requires that the specification teach those in the art to make and use the invention without "undue experimentation." *In re Wands,* 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). That *some* experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." *Id.* at 736-37, 8 USPQ2d at 1404. Enablement, like obviousness, is a question of law which we independently review, although based upon underlying factual findings which we review for clear error. *See id.* at 735, 8 USPQ2d at 1402.
In response to the § 112 rejection, appellants assert that their invention is "pioneering," and that this should entitle them to claims of broad scope. Narrower claims would provide no real protection, appellants argue, because the level of skill in this art is so high, art workers could easily avoid the claims. Given the disclosure in their specification, appellants contend that any skilled microbiologist could construct vectors and transform many different cyanobacteria, using a variety of promoters and *Bacillus* DNA, and could easily determine whether or not the active *Bacillus* protein was successfully expressed by the cyanobacteria.
The PTO made no finding on whether the claimed invention is indeed "pioneering," and we need not address the issue here. With the exception of claims 47 and 48, the claims rejected under § 112 are not limited to any particular genus or species of cyanobacteria. The PTO's position is that the cyanobacteria are a diverse and relatively poorly studied group of organisms, comprising some 150 different genera, and that heterologous gene expression in cyanobacteria is "unpredictable." Appellants have not effectively disputed these assertions. Moreover, we note that only one particular species of cyanobacteria is employed in the working examples of appellants' specification, and only nine genera of cyanobacteria are mentioned in the entire document.
[4] Taking into account the relatively incomplete understanding of the biology of cyanobacteria as of appellants' filing date, as well as the limited disclosure by appellants of particular cyanobacterial genera operative in the claimed invention, we are not persuaded that the PTO erred in rejecting claims 1-46 and 50-51 under § 112, first paragraph. There is no reasonable correlation between the narrow disclosure in appellants' specification and the broad scope of protection sought in the claims encompassing gene expression in any and all cyanobacteria. *See In re Fisher,* 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970) (the first paragraph of § 112 requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification).

22 Accordingly, we affirm the § 112 rejection as to those claims.
In so doing we do *not* imply that patent applicants in art areas currently denominated as "unpredictable" must never be allowed generic claims encompassing more than the particular species disclosed in their specification. It

is well settled that patent applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art. *In re Angstadt,* 537 F.2d 498, 502-03, 190 USPQ 214, 218 (CCPA 1976). However, there must be sufficient disclosure, either through illustrative examples or terminology, 23 to teach those of ordinary skill how to make and how to use the invention as broadly as it is claimed. This means that the disclosure must adequately guide the art worker to determine, without undue experimentation, which species among all those encompassed by the claimed genus possess the disclosed utility. Where, as here, a claimed genus represents a diverse and relatively poorly understood group of microorganisms, the required level of disclosure will be greater than, for example, the disclosure of an invention involving a "predictable" factor such as a mechanical or electrical element. *See Fisher,* 427 F.2d at 839, 166 USPQ at 24. In this case, we agree with the PTO that appellants' limited disclosure does not enable one of ordinary skill to make and use the invention as now recited in claims 1-46 and 50-51 without undue experimentation.

Remaining dependent claim 47 recites a cyanobacterium which expresses the chimeric gene of claim 1, wherein the cyanobacterium is selected from among the genera *Anacystis* and *Synechocystis.* Claim 48, which depend from claim 47, is limited to the cyanobacterium *Synechocystis* 6803. The PTO did not separately address these claims, nor indicate why they should be treated in the same manner as the claims encompassing all types of cyanobacteria. Although these claims are not limited to expression of genes encoding particular *Bacillus* proteins, we note what appears to be an extensive understanding in the prior art of the numerous *Bacillus* proteins having toxicity to various insects. The rejection of claims 47-48 under § 112 will not be sustained.

CONCLUSION

The rejection of claims 1-48 and 50-52 under 35 USC 103 is *reversed.* The rejection of claims 1-46 and 50-51 under 35 USC 112, first paragraph, is *affirmed* and the rejection of claims 47 and 48 thereunder is *reversed.*

AFFIRMED-IN-PART, REVERSED-IN-PART

Footnotes

Footnote 1. Basic vocabulary and techniques for gene cloning and expression have been described in *In re O'Farrell,* 853 F.2d 894, 895-99, 7 USPQ2d 1673, 1674-77 (Fed. Cir. 1988), and are not repeated here.

Footnote 2. All living cells can be classified into one of two broad groups, procaryotes and eucaryotes. The procaryotes comprise organisms formed of cells that do not have a distinct nucleus; their DNA floats throughout the cellular cytoplasm. In contrast, the cells of eucaryotic organisms such as man, other animals, plants, protozoa, algae and yeast have a distinct nucleus wherein their DNA resides.

Footnote 3. "Transformed" cyanobacteria are those that have successfully taken up the foreign *Bacillus* DNA such that the DNA information has become a permanent part of the host cyanobacteria, to be replicated as new cyanobacteria are generated.

Footnote 4. "Expression" of a gene refers to the production of the protein which the gene encodes; more specifically, it is the process of transferring information from a gene (which consists of DNA) via messenger RNA to ribosomes where a specific protein is made.

Footnote 5. In the context of the claimed invention, "selectable markers" or "marker genes" refer to antibiotic-resistance conferring DNA fragments, attached to the gene being expressed, which facilitate the selection of successfully transformed cyanobacteria.

Footnote 6. *Nucleic Acids Res.* 8917 (1984).

Footnote 7. Chloramphenicol is an antibiotic; CAT is an enzyme which destroys chloramphenicol and thus imparts resistance thereto.

Footnote 8. *Biochem. and Biophys. Res. Comm.* 748 (1986).

Footnote 9. *Gene* 151 (1985).

Footnote 10. *Mol. Gen. Genet.* 181 (1983).
Footnote 11. *Mol. Gen. Genet.* 505 (1986).
Footnote 12. *J. Bacteriology* 246 (1979).
Footnote 13. RNA polymerase, the enzyme responsible for making RNA from DNA, binds at specific nucleotide sequences (promoters) in front of genes in DNA, and then moves through the gene making an RNA molecule that includes the information contained in the gene. Initiation specificity is the ability of the RNA polymerase to initiate this process specifically at a site(s) on the DNA template.
Footnote 14. *Proc. Natl. Acad. Sci. USA* 5961 (1984).
Footnote 15. *Mol. Gen. Genet.* 185 (1986).
Footnote 16. *Gene* 211 (1984).
Footnote 17. *Gene* 289 (1984).
Footnote 18. Denotes different species or organism.
Footnote 19. MPEP 706.03(n), "Correspondence of Claim and Disclosure," provides in part:
In chemical cases, a claim may be so broad as to not be supported by [the] disclosure, in which case it is rejected as unwarranted by the disclosure....
Footnote 20. MPEP 796.03(z), "Undue Breadth," provides in part:
n applications directed to intentions in arts were the results are unpredictable, the disclosure of a single species usually does not provide an adequate basis to support generic claims. *In re Sol,* 1938 C.D. 723; 497 O.G. 546. This is because in arts such as chemistry it is not obvious from the disclosure of one species, what other species will work. *In re Dreshfield,* 1940 C.D. 351; 518 O.G. 255 gives this general rule: "It is well settled that in cases involving chemicals and chemical compounds, which differ radically in their properties it must appear in an applicant's specification either by the enumeration of a sufficient number of the members of a group or by other appropriate language, that the chemicals or chemical combinations included in the claims are capable of accomplishing the desired result." ...
Footnote 21. *Stedman's Medical Dictionary* 1139 (24th ed. 1982) (definition of "Procaryotae"). Procaryotic organisms are commonly classified according to the following taxonomic hierarchy: Kingdom; Division; Class; Order; Family; Genus; Species. 3 *Bergey's Manual of Systematic Bacteriology* 1601 (1989).
Footnote 22. The enablement rejection in this case was not based upon a post-filing date state of the art, as in *In re Hogan,* 559 F.2d 595, 605-07, 194 USPQ 527, 536-38 (CCPA 1977). *See also United States Steel Corp. v. Phillips Petroleum Co.,* 865 F.2d 1247, 1251, 9 USPQ2d 1461, 1464 (Fed. Cir. 1989) (citing *Hogan*); *Hormone Research Found., Inc. v. Genentech, Inc.,* 904 F.2d 1558, 1568-69, 15 USPQ2d 1039, 1047-48 (Fed. Cir. 1990) (directing district court, on remand, to consider effect of *Hogan* and *United States Steel* on the enablement analysis of *Fisher*), *cert. dismissed,* — U.S. —, 111 S. Ct. 1434 (1991). We therefore do not consider the effect of *Hogan* and its progeny on *Fisher* 's analysis of when an inventor should be allowed to "dominate the future patentable inventions of others." *Fisher,* 427 F.2d at 839, 166 USPQ at 24.
Footnote 23. The first paragraph of § 112 requires nothing more than *objective* enablement. *In re Marzocchi,* 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is irrelevant. *Id.*

Dissenting Opinion Text

Dissent By:

Mayer, J., dissenting.

An appeal is not a second opportunity to try a case or prosecute a patent application, and we should not allow parties to "undertake to retry the entire case on appeal." *Perini America, Inc. v. Paper Converting Machine Co.,* 832 F.2d 581, 584, 4 USPQ2d 1621, 1624 (Fed. Cir. 1987); *Eaton Corp. v. Appliance Valves Corp.,* 790 F.2d 874,

877, 229 USPQ 668, 671 (Fed. Cir. 1986). But that is precisely what the court has permitted here. The PTO conducted a thorough examination of the prior art surrounding this patent application and concluded the claims would have been obvious. The board's decision based on the examiner's answer which comprehensively explains the rejection is persuasive and shows how the evidence supports the legal conclusion that the claims would have been obvious. Yet, the court ignores all this and conducts its own examination, if you will, as though the examiner and board did not exist. Even if thought this opinion were more persuasive than the board's, I could not join it because it misperceives the role of the court.

The scope and content of the prior art, the similarity between the prior art and the claims, the level of ordinary skill in the art, and what the prior art teaches are all questions of fact. *Graham v. John Deere Co.,* 383 U.S. 1, 17, 148 USPQ 459, 467 (1966); *Jurgens v. McKasy,* 927 F.2d 1552, 1560, 18 USPQ2d 1031, 1037 (Fed. Cir. 1991). And "[w]here there are two permissible views of

the evidence, the factfinder's choice between them cannot be clearly erroneous." *Anderson v. City of Bessemer City,* 470 U.S. 564, 574 (1985). The mere denomination of obviousness as a question of law does not give the court license to decide the factual matters afresh and ignore the requirement that they be respected unless clearly erroneous. *In re Woodruff,* 919 F.2d 1575, 1577, 16 USPQ2d 1934, 1935 (Fed. Cir. 1990); *In re Kulling,* 897 F.2d 1147, 1149, 14 USPQ2d 1056, 1057 (Fed. Cir. 1990). There may be more than one way to look at the prior art, but on this record we are bound by the PTO's interpretation of the evidence because it is not clearly erroneous and its conclusion is unassailable. I would affirm on that basis.

- End of Case -

Review

Genetic Transformation of Yeast

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ABSTRACT

Genetic transformation was first described by Griffith in 1928 and has since been demonstrated in a variety of organisms, including many species of fungi. This review focuses on the history and technology of the transformation of Saccharomyces cerevisiae. *The application of protocols developed for* S. cerevisiae *to other important yeast species is discussed. The protocols for transformation by spheroplasting, LiAc/ssDNA/PEG, and electroporation are compared, and possible mechanisms for transformation are discussed.*

INTRODUCTION

The term "transformation" was first used by Griffith (50) to describe heritable changes in certain characteristics of *Pneumococcus* and is now used to describe DNA uptake in both prokaryotes and eukaryotes, as detected by consequent changes in phenotype. In his extensive and elegant study, Griffith (50) showed that a non-reverting avirulent form of *Pneumococcus* that lacked a polysaccharide capsule could be "transformed" to a virulent form by injecting heat-killed encapsulated virulent bacteria along with the non-virulent inoculum into the peritoneal cavity of a mouse. He suggested that the "transforming principle" might be a component of the polysaccharide capsule.

In a landmark study, the transforming factor was purified and found to be DNA by Avery, McLeod, and McCarty (3). After its initial description, DNA transformation was attempted for various organisms. These included a number of different yeasts (73,92), *Neurospora crassa* (84), and *Drosophila melanogaster* (38). However, until molecular cloning became available to provide evidence of DNA transfer, there were skeptics (53). The ability to clone eukaryotic DNA fragments in *E. coli* (20,71,87) was an important milestone in the development of transformation protocols for all organisms. The development of molecular genetics in all experimental systems, from bacteria to mammals, has been catalyzed by the ability to induce DNA uptake and subsequent expression in living cells.

Genetic transformation of *Saccharomyces cerevisiae*, was first reported by Oppenoorth (92) in 1960; however, other workers were unable to repeat his results (53). Khan and Sen (73) carried out an extensive study of DNA transformation of various genetic markers with a number of different yeast species. Their procedure was to grow yeast cultures in the presence of DNA extracted from various yeast strains and then to screen for colonies with a transformed phenotype. They investigated the effects of DNA concentration and the age of cells for best transformation. Their studies furnished convincing evidence for the transformation of yeast but failed to stimulate much interest in the phenomenon.

In 1989, Fincham (37) published a comprehensive review of fungal transformation, which discussed transformation technology and mechanisms of plasmid integration and expression to that date. This review focuses on the development and improvements to the various techniques employed to achieve genetic transformation of yeast.

THE SPHEROPLAST METHOD

The removal of the yeast cell wall by enyzmatic treatment to yield protoplasts was first reported by Eddy and Williamson (35) in 1957, based on the observation by Giaja that the "the gut juice of the snail *Helix pomatia* dissolves the cell wall of whole yeasts". Protoplasts, produced by the treatment of yeast cells with snail enzyme, regenerated when embedded in medium containing 30% gelatin (113) or 2% agar (112). Hutchison and Hartwell (60) noted that treatment of yeast cells with Glusulase (commercial snail enzyme) did not remove the cell wall completely and suggested that cells produced in this way be termed "spheroplasts". In 1974, Kao and Michayluk (68) showed that polyethylene glycol (PEG) stimulated

the fusion of plant protoplasts. van Solingen and van der Plaat (117) used PEG plus $CaCl_2$ treatment to fuse yeast spheroplasts derived from strains of the same mating type but with complementary nutritional requirements. This experimental procedure resulted in the exchange of genetic information in yeast by a nonsexual process and could be considered analogous to the soon-to-be-discovered process of transformation.

In 1977, Ratzkin and Carbon (98) reported the isolation of specific hybrid plasmids, constructed from an *E. coli* plasmid and fragments of yeast DNA, which complemented the *hisB* and *leuB* mutations in *E. coli*. The first successful protocol for the genetic transformation of *S. cerevisiae*, developed by Hinnen et al. (58), had its origins in the investigation of yeast spheroplasts (60) and spheroplast fusion (117). They used the pYEleu10 plasmid (98) containing the *LEU2* gene to transform a yeast *leu2 3-112* mutant to prototrophy. They removed the yeast cell wall enzymatically and stabilized the resulting spheroplasts with 1.0 M sorbitol before treating them with PEG and plasmid DNA. The treated yeast cells were then suspended in regeneration agar and plated onto medium to select for *LEU+* cells. More than 100 putative transformants were then analyzed by Southern blot analysis using the *E. coli* plasmid sequence as a probe. Three types of transformants were identified. In type I transformants, the plasmid DNA was integrated adjacent to the *leu2* locus. In type II transformants, the plasmid had integrated at other locations in the yeast genome. Type III transformants did not contain any bacterial DNA sequences. The authors conclude that the type III transformants were most likely due to replacement of the *leu2 3-112* allele with the *LEU2* sequence from the transforming plasmid. The transformation efficiency was in the range of 30–50 transformants/μg plasmid DNA. Because this plasmid did not contain a yeast replicon, integration was required for the transformants to be stable.

Five months later, Beggs (11) reported the transformation of yeast with an autonomously replicating plasmid. She constructed chimeric plasmids by inserting the endogenous autonomously replicating yeast 2-μm circle into the bacterial plasmid pMB9. These chimeric plasmids were then used to construct a yeast DNA library. Two plasmids from this library, pJDB248 and pJBD219, proved to complement the *leuB* mutation in *E. coli*. These plasmids were then used to transform a yeast strain containing the *leu2-3* mutant allele utilizing essentially the same protocol as that reported by Hinnen et al. (58). The *LEU+* transformants occurred at a frequency of 10^{-5} to 10^{-3} and an efficiency of 1×10^4 transformants/μg plasmid DNA. Southern blot analysis was used to confirm the presence of plasmid sequences, verifying that DNA uptake and genetic transformation had occurred. Finally, the plasmids were recovered from the transformed yeast cells by transformation back into *E. coli* and were shown to have a structure similar to the original plasmid construct.

Plasmid Vector Development

Three additional types of chimeric plasmid vectors were developed by Struhl et al. (111): *(i)* YIp (yeast integrating plasmids), which are unable to replicate and transform by integration into the genome of the recipient strain; *(ii)* YEp (yeast episomal plasmids), which carry the replication origin of the yeast 2-μm circle, an endogenous yeast plasmid, and can replicate in the recipient cell; and *(iii)* YRp (yeast replicating plasmids), which can replicate utilizing yeast autonomous replicating sequences (ARS). These authors showed that integrating vectors transformed with low efficiencies, 1–10 transformants/μg. Plasmids that could replicate in the yeast cell transformed with much higher efficiencies. The YEp vectors transformed with an efficiency of $0.5–2.0 \times 10^4$ transformants/μg input plasmid DNA, and the YRp7 plasmid produced $0.5–2.0 \times 10^3$ transformants/μg input plasmid DNA. Struhl et al. (111) demonstrated that plasmids that require integration into the genome transform less efficiently than those yeast plasmid vectors that can replicate autonomously in the yeast cell. Since then, two other yeast plasmid vectors have been developed. Yeast centromere plasmids (YCp) that carry an ARS and a yeast centromere (25,93) are more stable than YRp plasmids but are present in only one copy per cell. Yeast artificial chromosomes (YACs) are propagated as a circular plasmid with a centromere and an ARS plus two selectable markers, two telomeres, and a cloning site (17,88). The vector is linearized by the removal of a sequence between the telomeres, and foreign DNA is inserted into the cloning site. The result is a linear artificial chromosome, 100–1000 kb in length, that can be propagated through mitosis and meiosis.

Further Development of the Spheroplast Method

The spheroplasting method of transformation quickly became the gold standard for *S. cerevisiae*. However, Harashima (52) suggested that transformation or DNA uptake was associated with fusion of protoplasts generating polyploid cells during the process. Burgers and Percival (16) showed that cell fusion could be avoided by careful control of the spheroplasting and transformation conditions. They reported transformation efficiencies ranging from 0.4 to 3.0×10^7 transformants/μg input DNA, with ssDNA being 2- to 5-fold more efficient. This is the highest published transformation efficiency using this method, possibly due to the addition of calf thymus or *E. coli* carrier DNA to the transformation mixture, which may act to protect the plasmid DNA from nucleases (16).

An interesting technique for transforming yeast cells by fusing them with bacterial minicells was published by Gyuris and Duda (51). Bacterial minicells containing plasmid DNA were converted to protoplasts with lysozyme, mixed with yeast cell protoplasts, and treated with PEG to promote cell fusion. This procedure resulted in up to 10^7 transformants/μg plasmid DNA compared to 7×10^3 transformants/μg DNA in a control experiment using the spheroplast protocol.

Johnston et al. (65) showed that the spheroplast transformation efficiency displayed by any one strain was a complex genetic trait in *S. cerevisiae*. Crosses between strains that showed high and low transformation efficiencies suggested that the high-efficiency phenotype was inherited as a recessive polygenic trait. In addition, these

authors showed that incubation of spheroplasts with PEG and DNA at 4°C increased transformation efficiency approximately 5-fold for some strains. Increased transformation efficiency in response to a cold shock has also been noted by Broach et al. (12).

Although the spheroplast protocol has been used extensively, it suffers from the drawback that the transformed cells must be suspended and plated in regeneration agar. This means that the transformants cannot be replica plated easily and must be picked individually from the layer of regeneration agar. Substitution of agar by calcium alginate, which dries to form a very thin layer but allows cell wall regeneration (19), allows sampling by replica plating. With the development of other methods of transformation, the spheroplasting method is now mainly used for transformation of YACs (17). A protocol for spheroplast transformation described by Spencer et al. (110) is shown in Table 1.

Table 1. Spheroplast Transformation Protocol

1. Cells are grown in 50 mL YPAD to a density of 3×10^7 cells/mL.
2. The cells are harvested by centrifugation at 400–600× *g* for 5 min, washed twice in 20 mL sterile water, and washed once in 20 mL 1 M sorbitol. The cells are resuspend in 20 mL SPEM (1 M sorbitol, 10 mM sodium phosphate, pH 7.5, 10 mM EDTA plus 40 µL β-mercaptoethanol added immediately before use).
3. The cells are converted to spheroplasts by the addition of 45 µL zymolyase 20T (10 µg/mL) and incubation at 30°C for 20–30 min with gentle shaking. By this time, 90% of the cells should be converted to spheroplasts.
4. The spheroplasts are harvested by centrifugation at 250× *g* for 4 min, and the supernatant is removed carefully. The pellet is washed once in 20 mL STC (1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) and resuspended in 2 mL STC.
5. Spheroplasts are transformed by gently mixing 150 µL of the suspension in STC with 5 µg carrier DNA and up to 5 µg plasmid DNA in less than 10 µL. The mixture is incubated for 10 min at room temperature. One milliliter of PEG reagent [10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 20% (w/v) PEG 8000; filter sterile] is added and mixed gently, and incubation is continued for another 10 min.
6. The spheroplasts are harvested by centrifugation for 4 min at 250× *g* and resuspended in 150 µL SOS (1.0 M sorbitol, 6.5 mM $CaCl_2$, 0.25% yeast extract, 0.5% bactopeptone). Dilutions of spheroplasts are mixed with 8 mL TOP (selective medium containing 1.0 M sorbitol and 2.5% agar kept at 45°C) onto the appropriate selective medium containing 0.9 M sorbitol and 3% glucose. Transformants can be recovered after incubation for 3–4 days at 30°C.

INTACT YEAST CELL TRANSFORMATION

Kimura et al. (74) first reported the transformation of intact yeast cells in 1981. Cells were apparently transformed with plasmid vectors after treatment with Triton® X-100; unfortunately, no experimental details or data were given, and we have been unable to find further information on this work. Two years later, Iimura et al. (62) showed that intact yeast cells could be induced to take up the plasmid YRp7 by treatment with a 200 mM $CaCl_2$ solution. Transformants were recovered by plating onto directly selective medium. The transformation efficiency was between 50 and 200 transformants/µg plasmid DNA. Interestingly, these authors reported that treatment with PEG and $CaCl_2$ did not increase the yield of transformants.

Li^+ Transformation

In 1983, Ito et al. (63) published the next innovation in intact yeast cell transformation. They showed that specific monovalent alkali cations (Na^+, K^+, Rb^+, Cs^+, and Li^+) could be used in combination with PEG to stimulate plasmid DNA uptake by intact yeast cells. Cells were pretreated in 100 mM cation solution followed by the addition of plasmid DNA and PEG (MW 4000) to a final concentration of 35% (w/v) and then incubated for 1 h at 30°C. This was followed by a 5-min heat shock at 42°C, after which the cells plated directly onto selective medium. The authors optimized cation concentration, treatment time, cell concentration, and DNA concentration and showed that both PEG and heat shock were essential for transformation. The best results were obtained with Li acetate (LiAc). The yields of transformants, 450 transformants/µg plasmid DNA, were lower that those obtained with contemporary spheroplasting procedures, but Li^+ transformation was faster, simpler, and easier. A major advantage was that treated cells did not have to be plated in regeneration agar and could be sampled by replica plating. For these reasons, most laboratories working with yeast readily adopted Li^+ transformation.

Further Development of the Li^+ Transformation Method

Later in 1983, Ito et al. (64) reported several modifications to their original protocol. The transformation efficiency was increased to 1300 transformants/µg plasmid DNA if cells were treated for 1 h in the presence of 2-mercaptoethanol and then subjected to the original transformation protocol utilizing LiCl but omitting the heat shock. Dithiothreitol (DTT), L-cysteine, and reduced glutathione did not stimulate transformation in a fashion similar to 2-mercaptoethanol, but PEG was essential. In the same year, Klebe et al. (75) showed that PEG alone could be used to induce DNA uptake by both *E. coli* and yeast cells. Yeast cells that had been frozen and thawed were incubated with plasmid DNA and PEG1000 for 1 h at 30°C. The highest transformation efficiencies obtained were 150 transformants/µg, only 1.5% of those obtained by spheroplasting. Yamakawa et al. (119) also showed that PEG, in the absence of LiAc, could stimulate transformants. The transformation efficiency was only about a third of that seen when LiAc and PEG were used in combination. They also showed that transformation by this method varies from strain to strain, suggesting that the ability to transform by this method has a genetic component.

Over the next few years, a number of authors published variations on the

intact yeast cell transformation method. Bruschi et al. (14) also showed that Ca^{2+} ions could be used in place of Li^{+} ions and that strains varied in their responses to different cations. They suggested that PEG was responsible for the adsorption of the DNA to the yeast cell. Keszenman-Pereyra and Hieda (72) published a colony procedure for LiAc transformation; however, the transformation frequency was only about 1000 transformants/µg plasmid DNA with fresh colonies and lower with stored colonies. These authors also showed that single-stranded vector DNA did not transform as effectively as double-stranded vector DNA.

By 1987, the protocol for transforming intact yeast cells was standardized to include PEG, LiAc, and a heat shock. The addition of sonicated carrier DNA to the transformation increased the efficiency to 2×10^4 transformants/µg plasmid DNA (44,103). In 1989, Schiestl and Gietz (106) showed that the addition of single-stranded carrier DNA or RNA to the transformation reaction stimulated the production of transformants to reach 2×10^4 transformants/µg plasmid DNA/10^8 cells. Further studies (42) showed that this procedure was efficient enough to clone yeast genes, including *RAD4*, which is toxic in *E. coli*, directly from a library ligation reaction. If two vectors carrying different selectable markers were used in the transformation

Table 2. LiAc/ssDNA/PEG Protocol

1. Cells are grown overnight in 2× YPAD, resuspended at 5×10^6 cells/mL in warm 2× YPAD and regrown for two cell divisions to 2×10^7 cells/mL.
2. The cells are harvested by centrifugation at 3000× *g* for 5 min, washed twice in sterile distilled water, and resuspended in sterile distilled water at 10^9 cells/mL.
3. Samples of 10^8 cells are transferred to 1.5 mL microcentrifuge tubes, the cells are pelleted, and the supernatant are discarded.
4. The pellets are resuspended in 360 µL transformation mixture [240 µL 50% PEG 3500 (w/v), 36 µL 1.0 M LiAc, 50 µL 2.0 mg/mL single-stranded carrier DNA, 0.1–10 µg plasmid DNA plus water to 34 µL].
5. The cells in transformation mixture are incubated at 42°C for 40 min. The cells are pelleted in a microcentrifuge, and the transformation mixture is removed.
6. The cell pellet is gently resuspended in 1 mL sterile water, and samples are plated onto selective medium.

mixture, co-transformation occurred in 30%–40% of the cells selected for one of these genes. The high frequency of co-transformation could be utilized to knock out two different genes in a single transformation reaction or look for nonselective gene disruption using co-transformation with a selective plasmid (42).

Gietz et al. (41) reported that reducing the time that cells were incubated in TE/LiAc buffer improved the transformation efficiency to 1.0×10^6 transformants/μg plasmid DNA/10^8 cells. Omission of TE from the transformation buffer (46) and optimization of the cell number and carrier and plasmid DNA concentration in the transformation mixture (43) all added to the efficiency and reproducibility of this technique such that one can expect up to 5×10^6 transformants/μg plasmid DNA/10^8 cells with most strains (107). The LiAc/ssDNA/PEG protocol can also be carried out in microplates (47), allowing the simultaneous transformation of multiple strains or colonies. However, a microplate centrifuge is required, and a multichannel pipettor and a 96-prong replicator allow for greater efficiency.

The most recent and efficient version of the LiAc/ssDNA/PEG protocol (118) is shown in Table 2. This protocol should work well for most laboratory strains and is suitable for high-efficiency transformation of plasmid libraries for applications such as the yeast two-hybrid system (45).

A number of modifications of the original LiAc/ssDNA/PEG procedure have been published; some are specific for certain growth conditions, and others report an increase in the yields of transformants. For example, several groups report that adding DMSO (10%) to the transformation mixture increases yield (6,57). Similar improvements in the yield of transformants have been noted if ethanol is added to cells either before or during transformation (78). Soni et al. (109) characterized the effect that adding DMSO or ethanol or both to a transformation reaction utilizing both the rapid and high-efficiency protocols. They found that the increase in transformation efficiency was dependent on the transformation protocol and the specific strain of yeast. The most effective combination gave 50-fold increases in the yield of transformants. The strain-specific effects of ethanol and DMSO were not additive, and both reduced the viability of yeast cells.

Rapid transformation procedures have been published by Baker (4), Chen et al. (22), Schiestl et al. (107), Gietz and Woods (46,47), and Woods and Gietz (118). In Baker's protocol (4), the yeast cells are scraped from a plate and then treated with the LiAc, PEG, and double-stranded carrier DNA to give a transformation efficiency of 1.1×10^4 transformants/μg DNA. Freshly grown cells work best; however, four-week-old cultures also gave transformants, albeit at a greatly reduced frequency. Chen et al. (22) showed that cells in stationary phase treated with a "one-step" buffer containing LiAc, PEG, DTT, single-stranded carrier DNA, and plasmid DNA transformed at an efficiency of 2.1×10^4 transformants/μg plasmid DNA. Reddy and Maley (101) also included DTT in their transformation mixture and showed that, in combination with Ca^{2+}, it increased the yield of transformants about 30-fold to 2.0×10^4 transformants/μg DNA. The protocols from our laboratory (46,47,107,118) are all based on the LiAc/ssDNA/PEG procedure and yield up to 1×10^5 transformants/μg plasmid DNA with good transforming laboratory strains. Extension of the heat shock at 42°C up to 3 h increases the yield of transformants 10-fold in some strains (118). A modification of this approach for transforming many different strains at once with a single plasmid was developed by Elble (36). Essentially, the cells are resuspended in LiAc, PEG, and carrier DNA and left overnight at room temperature, and the transformed cells are then plated directly from this mixture onto selective media. The transformation levels range from 2.4×10^4 transformants for cells grown in culture to only 960 transformants/μg DNA when cells were harvested from a plate. This technique was also shown to work for *Schizosaccharomyces pombe* transformation giving rise to 2×10^4 transformants when liquid cultures were used and 208 transformants with a plate-grown cell sample.

Dohmen et al. (34) have developed a method for production of transformation competent frozen yeast cells that yields from 0.2 to 1.0×10^5 transformants/μg DNA. The procedure is also applicable to *Schwanniomyces occidentalis*, *S. pombe*, *Kluyveromyces lactis*, and *Hansenula polymorpha*, with transformation efficiencies between 50 and 1000 transformants/μg, depending on the plasmid and yeast species. Tan et al. (114) also published a method for the production of frozen yeast cells that can be used for transformation. The yield was about 1000 transformants/μg plasmid DNA when a single plasmid was used and about 150 transformants/μg when two plasmids were used. Both methods are good for producing competent yeast cells ahead of time and then doing transformations at a moment's notice.

In 1991, Moerschell et al. (85) described an LiAc transformation protocol for in vivo mutagenesis with synthetic oligonucleotides. Modifications of this technique (80,120) have improved the yields of transformants to give 50 transformants/μg when 100 μg oligonucleotide were used in the transformation mixture.

Transformation by Electroporation

Electroporation was first used by Neumann et al. (90) to induce DNA uptake and transformation in mouse cells. It is now widely used for the transformation of animal cells (23,55,96) and plant cells (8) and is the standard procedure for transforming bacteria in many laboratories (18,83). It is believed that the electric pulse results in the formation of transient pores in the cell membrane, allowing the entry of macromolecules (95). Electroporation was first applied to yeast spheroplasts by Karube et al. (69) and to intact yeast cells by Hashimoto et al. (54). Simpler protocols for the electroporation of intact yeast cells were subsequently reported by Delorme (33) and Simon and McEntee (108). Delorme (33) resuspended log phase cells in fresh medium and electroporated 0.1 μg plasmid DNA using 2.25 kV/cm and 25 μF; Simon and McEntee (108) resuspended washed late log phase cells in 0.5 mM Na phosphate buffer containing 10% glycerol and electroporated using 2.7 kV/cm and 10 μF. Both techniques produced 1–4.5×10^3 transformants/μg plasmid DNA. Rech et al. (100) reported that both circular and linear yeast artificial chromosomes could be electroporated into yeast cells with an

Table 3. Electroporation Protocol

1. Cells are grown in YPD to a density of 1×10^7 cells/mL.
2. The cells are harvested by centrifugation (1500× *g* for 5 min), resuspended at 1×10^9 cells/mL in 25 mM DTT (made in YPD medium, 20 mM HEPES, pH 8.0) and incubated for 10 min at 30°C.
3. The cells are then washed twice with EB (10 mM Tris-HCl, pH 7.5, 270 mM sucrose, 1 mM $MgCl_2$) and resuspended at 1×10^9 cells/mL in EB.
4. Samples of 48 µL are mixed with 2 µL plasmid DNA and delivered between the electrodes of a square pulse generator CNRS cell electropulsator.
5. The cells are pulsed with a field strength of 1.74 kV/cm and a pulse length of 15 ms.
6. One milliliter of prewarmed 30°C YPD is added immediately, and the suspension is incubated for 1 h at 30°C. The cells are then pelleted in a microcentrifuge resuspended in SD medium and plated onto the appropriate medium and incubated.

efficiency level of up to 325 transformants/µg. They used a modification of the Hashimoto et al. (54) procedure, which included PEG in the electroporation buffer. The PEG electroporation protocol can be applied to integrative transformation, giving a transformation efficiency of up to 1.8×10^3 transformants/µg (56). Becker and Guarente (10) reported that cells electroporated in 1.0 M sorbitol at 7.5 kV/cm and a 25 µF capacitance with a 200 Ω resistance in parallel with the sample and then plated onto selective medium containing 1.0 M sorbitol gave transformation efficiencies greater that 3.0×10^5 transformants/µg plasmid DNA. In 1992, Grey and Brendel (49) reported a rapid colony procedure for electroporation. Yeast cells were scraped from plates and electroporated in HEPES buffer with up to 1 µg DNA using a Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA, USA) with 1.4 kV, 25 µF, and 200 Ω in a 0.2-cm gap electroporation cuvette. Transformation efficiencies ranged from 2 to 8×10^3 transformants/µg.

Meilhoc et al. (82) reported that electroporation of yeast could be achieved utilizing a square pulse generator CNRS cell electropulsator (JOUAN-France) with a field strength of 1.74 kV/cm. The cell suspension is introduced between two parallel stainless steel flat electrodes 0.5 cm in width and 0.3 cm apart in a petri dish (39,40). They also showed that replicating cells in early or mid log phase are transformed 10 times more efficiently than stationary cells using this method. In addition, incubating yeast cells in 25 mM DTT for 10 min increased the electroporation efficiency up to 5-fold. This procedure can also be applied to frozen yeast cells with highly efficient results.

Manivasakam and Schiestl (81) reported that a hybrid technique using elements of both the LiAc/ssDNA/PEG and electroporation protocols that resulted in 5.2×10^6 transformants/µg plasmid DNA. Yeast cells were grown overnight to a density of $1–2 \times 10^8$ cells/mL and resuspended in a 100-µL volume containing 1.0×10^{10} cells. The cells were then incubated with 20 µg ssDNA, PEG, and plasmid DNA at 42°C for 15 min. The cells were electroporated in a 0.2-cm cuvette using a Gene Pulser set at 1.5 kV, 25 µF, with a pulse controller at 200 Ω, diluted in ice-cold YPAD, and plated. The best efficiencies were obtained with 34–170 ng plasmid DNA. However, transformation efficiency decreased with higher plasmid DNA concentrations.

More recently, Thompson et al. (115) reported that yeast cells pretreated in a LiAc/DTT/TE buffer, washed and then electroporated in 1.0 M sorbitol, gave transformation efficiencies of $0.5–1.4 \times 10^6$ transformants/µg plasmid DNA. This pretreatment increased the yield of transformants 15- to 300-fold for strains that were refractory to conventional electroporation. The conditions for electroporation were 1.5 kV, 25 µF, and 200 Ω with the Gene Pulser.

Electroporation has also been used to introduce oligonucleotides into yeast using both the exponential (120) and

Table 4. Advantages and Disadvantages of Transformation Methods

Technique	Advantages	Disadvantages
Spheroplasting	• Efficient transformation of large DNA constructs such as YACs • No special equipment needed • Low cost	• Difficult to optimize • Cells must be grown overnight. • Lengthy protocol • Cell must be plated on osmotically neutral supportive medium. • Strain specific variation in transformation efficiencies
LiAc/ssDNA/PEG	• Most efficient procedure (except for large DNA constructs) • No special equipment needed • Simple protocol • Easily optimized • Frozen cells can be used. • Low cost	• Cells must be grown overnight (unless frozen cells available). • Strain specific variation in transformation efficiencies
Electroporation	• Efficient procedure (except for large DNA constructs) • Short protocol • Easily optimized • Frozen cells can be used.	• Substantial setup cost • Cells must be grown overnight (unless frozen cells available). • Strain specific variation in transformation efficiencies
Glass Bead method	• Simple protocol • Low cost	• Low efficiency transformation • Cells need osmotic support.
Biolistic transformation	• Relatively efficient transformation • Mitochondria can be transformed.	• High setup cost • Difficult to determine efficiency • Preparation of projectiles necessary

square wave formats (5). Square wave electroporation gave an optimal efficiency of 80 transformants/µg oligonucleotide. The low efficiency of this type of transformation may in part be due to the requirement for replacement of the chromosomal sequence by that of the oligonucleotide. Yamamoto et al. (120) reported that oligonucleotide transformation in a *rad52* strain was more efficient than transformation with linearized integrating plasmid and suggested that the mechanism of transformation by oligonucleotides differs from that of dsDNA.

Electroporation protocols require the delivery of an electric pulse to cells, either in an electroporation cuvette (10,49,56,81,100,108,115) or between electrodes in a petri dish (39,40,82). The parameters of the electroporation: field strength (kV/cm), capacitance (µF), and resistance (Ω), are different in each protocol, as are the specifics of the preparation of cells, and it is clear that there is considerable variation in the yield of transformants between yeast strains. For any given yeast strain, it may be necessary to investigate the parameters of the pulse and the treatment of cells to obtain a sufficient number of transformants. Electroporation takes less time than either LiAc/ssDNA/PEG or spheroplast transformation, but the initial cost is higher. An efficient electroporation protocol (82) is shown in Table 3.

Transformation Using Glass Beads

In 1988, Costanzo and Fox (27) reported transformation of yeast cells by agitation with glass beads in the presence of carrier and plasmid DNA. The best transformation efficiency, approximately 300 transformants/µg, was obtained when 2 mL cells were harvested in late log phase, washed twice and resuspended in 1/10 volume of selective medium containing 1.0 M sorbitol. Carrier DNA, 50 µg dissolved in selective medium containing 1.0 M sorbitol, and 5 µg plasmid DNA were added to the cell suspension. Sterile glass beads (0.3 g; 0.45–0.52 mm diameter) were added, and the mixture was vortex mixed at top speed for 15–45 s. The supernatant containing the cells was plated onto selective medium containing 1.0 M sorbitol. The authors note that no transformants are recovered in the absence of sorbitol in the plating medium and suggest that all transformants are derived from the small fraction of cells that are too badly damaged to survive unless osmotically stabilized by sorbitol. This method rivals electroporation for being the least time consuming but is also one of the least efficient of those in current use.

Biolistic Transformation of Yeast

The biolistic method of transformation was first developed for plant cells (77) and was successfully applied to yeast in 1988 (66). The yeast cells are plated onto the selective medium and then bombarded with 0.5 µm gold or tungsten DNA-coated projectiles using compressed helium (2). The plates are then incubated at 30°C to allow the growth of transformants. A single bombardment usually gives rise to a plate confluent with transformants (67). This translates to a transformation efficiency of 10^4 to 10^5 transformants/µg plasmid DNA. Transformation of mitochondrial markers can be accomplished with pro-

jectiles coated with mitochondrial genes at 0.1% of the rate of nuclear transformation. This is the only protocol that can accomplish transformation for yeast mitochondrial genes. The physical nature of this technique is not dependent on cellular characteristics (composition of the cell wall and membrane), and strains or species of yeast that are refractory to conventional techniques should yield transformants with this method. A commercial unit is available from Bio-Rad Laboratories.

TRANSFORMATION OF OTHER YEAST SPECIES

Transformation protocols have been developed for numerous other species of yeast of genetic or commercial importance. The following list is not exhaustive but will give readers a starting place to identify protocols for their specific needs.

S. pombe

Shortly after the discovery of spheroplast transformation in *S. cerevisiae* (11,58), this technique was applied to *S. pombe* (9). A protocol for this method, which yields $1–5 \times 10^4$ transformants/µg plasmid DNA, is given by Moreno et al. (86). Variants of this technique can be found on both the Forsberg laboratory Web site (pingu.salk.edu/~forsberg/tfmn.html) and the Fission Yeast Handbook from the Nurse Laboratory (www.bio.uva.nl/pombe/handbook/section2/section2-2.html). Protocols for the transformation of intact cells using Li^+ have also been developed. In 1987, Broker (13) characterized a number of variables and was able to generate $4–9 \times 10^3$ transformants/µg plasmid DNA. Okazaki et al. (91) published a method that improved the transformation efficiency up to 2×10^6 transformants/µg. Current protocols for the LiAc procedure can also be found at the Web sites listed above and in a recent paper by Chua et al. (24). Finally, Hood and Stachow (59) and Prentice (97) have published protocols for the electroporation of *S. pombe*. Transformation efficiencies ranged from 1×10^3 to 2×10^5 transformants/µg DNA. Protocols for electroporation can also be found at the Web sites listed.

Candida albicans

The transformation of this yeast can be accomplished using the LiAc or electroporation methods. Sanglard et al. (105) published a method that yields 50–100 transformants/µg linear plasmid DNA. Thompson et al. (115) found that pretreating cells with LiAc and DTT increased electroporation efficiencies 3- to 5-fold. De Backer et al. (31) published an electroporation protocol that yielded up to 300 transformants/µg integrating plasmid and 4500 transformants/µg *Candida* ARS (CARS)-containing plasmid. Other species of *Candida* can be transformed with modifications of the LiAc protocol (70).

Pichia pastoris

This methylotrophic yeast is currently being used to produce recombinant proteins. Specific expression vectors, containing the methanol-responsive promoter of the alcohol oxidase gene, give very high levels of recombinant proteins (28,104). Both the spheroplasting and electroporation methods can be used to transform this species. Methods for the transformation of this yeast species can be found in Cregg et al. (28) (spheroplasting), Rosenfeld (104) (electroporation), and Cregg and Russell (29) (both). In addition, a manual for spheroplast transformation can be found at the Invitrogen Web site (http://www.invitrogen.com/pdf_manuals/pichspher_man.pdf).

Other Yeast Species

The spheroplast, lithium cation, and electroporation transformation methods have been successfully applied to a number of different yeast species. These include *Hansenula polymorpha* (34,48,102,116), Klyveromyces *spp* (7, 30,34,61), *Yamadazyma ohmeri* (94), *Yarrowia lipolytica* (21), and *Schwanniomyces occidentalis* (26,34,76).

MECHANISMS OF TRANSFORMATION

The mechanisms by which DNA is transported into the cell, moves to the nucleus, and becomes established are

currently unknown. In the spheroplast method, the cell wall, which is presumed to act as a barrier to DNA, is removed before the cells are incubated with PEG and DNA. PEG has been shown to deposit plasmid and carrier DNA onto yeast cells (43). PEG treatment of spheroplasts probably results in the deposition of plasmid and carrier DNA onto the spheroplast surface. The mechanisms by which the plasmid DNA traverses the cell membrane and is transported to the nucleus and established have not been studied.

In the LiAc method, the cell wall is present and will act as a barrier to DNA. De Nobel and Barnett (32) consider the yeast cell wall to act as an ion exchanger. Gietz et al. (43) have proposed that the yeast cell wall binds ds and ssDNA molecules. They suggest that in the absence of carrier DNA most plasmid molecules are bound to the cell wall, making them unavailable for uptake and transformation. The addition of carrier DNA, ds or ss, saturates the cell wall DNA binding sites and increases the probability of plasmid DNA uptake. They propose that single-stranded carrier DNA gives higher yields of transformants because it does not compete with the double-stranded plasmid DNA for uptake and binds more effectively to the cell wall. This is consistent with the observation of Burgers and Percival (16) that single-stranded vector DNA transforms 2-fold more efficiently than the dsDNA using the spheroplasting technique, whereas single-stranded vectors transform 1000-fold less efficiently with the LiAc/ssDNA/PEG method (43,72).

Treatment of yeast cells with LiAc and β-mercaptoethanol has been shown to increase their permeability (15). Others have shown that the addition of DMSO (6,57,109) or ethanol (78,109) or DTT (22,101) increases transformation efficiency. We suggest that these treatments increase the permeability of the cell wall, in a manner analogous to the effects of an electric pulse (40), and may also affect the physiological properties of the cell membrane, in consequence increasing the uptake of plasmid DNA into the cytoplasm.

The yield of transformants obtained with cells grown to stationary phase (and beyond) can be increased to more than $1 \times 10^6/\mu g$ plasmid/10^8 cells by extending the duration of the incubation at 42°C (118). This suggests that the effects of incubation at 42°C are not related to the heat shock response (79) but rather that PEG acts as a solvent and extracts a component or components of the yeast cell wall. We suspect that DMSO and ethanol added to the transformation mixture increase this activity.

Recent studies have shown that plasmid topology has a significant effect on transformation efficiency. Raymond et

al. (99) constructed a plasmid with a 40-bp direct repeat on either side of a *Not*I restriction site and compared the results of transforming yeast with various topologies of this plasmid. Their results clearly showed that linear plasmids with terminal direct repeats transformed 2.5- to 5-fold more efficiently than either a circular plasmid with direct repeats, a linear plasmid with internal direct repeats, or a linear plasmid with a single terminal copy of the repeat sequence. This increase was obtained with both the electroporation protocol of Meilhoc et al. (82) and the LiAc/ssDNA/PEG protocol (1). These results suggest that linear DNA molecules are taken up more efficiently than circular molecules but that they require re-cirularization by homologous recombination or illegitimate end joining for transformation. Further research will be required to elucidate the specific details of transformation in yeast.

Interestingly, more studies have been devoted to the mechanism of transformation by electroporation in yeast than to either spheroplasting or the LiAc procedure. Ganeva et al. (39) have shown that the primary effect of the electric pulse is to induce transient changes in the cell envelope that allows macromolecules to enter and become trapped in the cell envelope. They found that a fluoresceinated dextran (FD70) became bound within the cell envelope after an EP pulse or treatment of cells with DTT. The pulse also affects the membrane, as evidenced by increased cytoplasmic accumulation of propidium iodide. Permeabilization to propidium iodide was accompanied by loss of viability, but a fraction of the permeabilized cells remained viable. These experiments did not involve transformation with DNA, but the results suggest that the viable permeabilized cells would be transformable. Subsequently, Ganeva et al. (40) carried out an analogous set of experiments with plasmid DNA. They showed that addition of DNase 2–3 s before EP completely prevented transformation; DNase added immediately after EP also reduced transformation, whereas, if the enzyme was added more that 7 s after EP, the yield was not affected. They proposed that transformation by electroporation involves the following series of events: *(i)* DNA in the transformation mixture accumulates on the cell surface, *(ii)* an electric pulse induces transient alterations in the cell envelope that allow insertion of plasmid DNA into the envelope and, perhaps, into the membrane, and *(iii)* removal of the field results in transfer of the DNA molecules across the membrane into the cytoplasm. They note that the movement of DNA across the envelope and the membrane is "mediated by the yeast cell" and is not the result of electrophoresis or free diffusion. Another model is presented by Neumann et al. (89). In a highly technical paper, these authors hypothesize from kinetic data that DNA uptake is a coupling of DNA binding and electrodiffusive penetration into the cell membrane. The DNA is then translocated across the membrane and binds to a cellular component, which then is the starting point for the transformation process. Further investigation is necessary to identify the cell components taking part in this process.

CONCLUDING REMARKS

The transformation techniques discussed in this review allow the recovery of up to 2.2×10^7 transformants/μg plasmid DNA (46). Each technique has advantages and disadvantages (Table 4), and the one best suited for a particular study will depend on the specific needs and resources of the individual researcher.

We have noted that little is known about the mechanisms of transformation in yeast. At the high efficiencies, it is only possible to recover about 5% transformed yeast cells. What are the specific qualities that characterize these "transformable" cells? We have isolated mutants that affect the efficiency of transformation by the LiAc/ssDNA/PEG method. Analysis of these mutants will allow the identification and characterization of genes involved in the transformation process and should elucidate the mechanisms of DNA uptake and establishment.

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Factors Affecting the Mitotic Stability of High-copy-number Integration into the Ribosomal DNA of *Saccharomyces cerevisiae*

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Yeast vectors suitable for high-level expression of heterologous proteins should combine a high copy number with high mitotic stability. The pMIRY integrative vector system, based upon targeted integration into the yeast rDNA locus, developed in our laboratory satisfies these criteria. However, insertion of a (foreign) gene drastically reduced its mitotic stability of the resulting vector in comparison to its parent. In this paper we have investigated a number of possible reasons for this reduction in stability. The results demonstrate that plasmid size is an important, but not the only, determinant of mitotic stability. Stable maintenance is only observed when the complete plasmid has a size no larger than that of the rDNA unit (9·1 kb). In addition stability depends upon the nature of the rDNA fragment present in the plasmid, required for targeting its integration. On the other hand, it turned out to be irrelevant for mitotic stability whether the heterologous gene was expressed or not. These findings will be important in the design of a pMIRY vector suitable for industrial production of heterologous proteins.

KEY WORDS — *Saccharomyces cerevisiae*; ribosomal DNA; plasmid; mitotic stability; heterologous protein

INTRODUCTION

Two important criteria to be met in order to make industrial production of a protein by means of recombinant DNA technology economically feasible are: (1) a high copy number of the gene introduced into the host organism in order to obtain a high-level expression and (2) a high mitotic stability of that gene, since the large-scale production of proteins often requires long-term culturing of the genetically modified micro-organism in non-selective media.

The most commonly used expression vectors in yeast are the episomal (YEp) plasmids which can be present in up to 200 copies per haploid genome (Broach, 1983). However, they require selection pressure for stable maintenance during long-term culturing (Walmsley *et al.*, 1983; Som *et al.*, 1988). Another type of expression vectors are the yeast integrating plasmids (YIp), which are integrated into the yeast genome by homologous recombination. These vectors are stably maintained but are usually present in a low copy number (1–5; Orr-Weaver and Szostak, 1983). We have recently developed a novel type of vector called pMIRY2 (for Multiple Integration in the Ribosomal DNA of Yeast) that integrates into the ribosomal DNA locus of *Saccharomyces cerevisiae* in up to 140 copies which are stably maintained over long periods of growth under non-selective conditions (Lopes *et al.*, 1989). Introduction of homologous or heterologous genes by means of this vector led to protein levels similar to those observed using a YEp vector of comparable copy number (Lopes *et al.*, 1989). Therefore pMIRY2 is a potentially very useful vector for biotechnological application. In this paper we describe studies aimed at establishing whether pMIRY-type plasmids can indeed be stably maintained under the conditions applied during industrial production of proteins.

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MATERIALS AND METHODS

Strains, transformants and culture conditions

Plasmids were constructed and isolated using *Escherichia coli* DH1 (Hanahan, 1982) and *E. coli* JM83 (Vieira and Messing, 1982) as hosts for $CaCl_2$- (Maniatis *et al.*, 1982) or RbCl- (Lopes *et al.*, 1989) mediated transformation.

S. cerevisiae YT6-2-1L (*cir*^{0}, *Mata*, *Leu2-3, 112, his4-519, can1*; Erhart and Hollenberg, 1981) was transformed using various procedures described by Beggs (1978), Carter *et al.* (1988) and Klebe *et al.* (1983). Yeast transformants were selected by plating on agar plates containing 0·67% (w/v) yeast nitrogen base (without amino acids), 2% (w/v) glucose, 0·01% (w/v) histidine. The same medium was used for growing the transformants in liquid culture. Total DNA was isolated from *S. cerevisiae* as described by Pedersen (1983).

Vector constructions

Plasmid pMIRY2 has been described previously in Lopes *et al.* (1989). This plasmid is basically composed of a 4·5 kb *Bgl*II-B fragment of yeast rDNA (comprising part of the 26S rRNA-gene, NTS1, 5S RNA-gene and NTS2: see Figure 1), a 450 bp chloroplast DNA marker fragment from *Spirodela oligohriza*, the *LEU2*d gene and pBR322 sequences, stiil containing the Amp^R-gene (see Figure 1). Plasmids pMIRY2D, pMIRY4, pMIRY2-Tp and pMIRY2-SOD are derivatives of pMIRY2 (Figure 1). pMIRY2D was derived by cutting pMIRY2 with *Sph*I followed by religation. This procedure removes 3·6 kb of the *Bgl*II-B rDNA fragment. In pMIRY4 the 585 bp *Bgl*II-*Hin*dIII fragment containing the chloroplast marker DNA has been replaced by a 34 bp synthetic linker having *Bam*HI, *Sal*I and *Xba*I sites. The entire homologous Mn^{2+} superoxide dismutase (SOD) gene (Marres *et al.*, 1985) comprised in a 2·1 kb *Bam*HI fragment was inserted into the unique *Bam*HI site of either pMIRY2 or pMIRY2D. The resulting plasmids were named pMIRY2-SOD and pMIRY2D-SOD, respectively. Plasmid pMIRY2D-Thau was constructed by insertion of a 2·1 kb *Bgl*II-*Bam*HI fragment comprising the coding region of the preprothaumatin gene (sweet-tasting protein from *Thaumatococcus danielli*; Edens and van der Wel, 1985) under control of the *GAPDH* promoter (Edens *et al.*, 1984) into the *Bgl*II site of pMIRY2D.

For the construction of pMIRY2-Tp, first a *Sac*I linker was inserted into the unique *Sma*I site of pMIRY2. Then the 963 bp *Sac*I-*Hin*dIII pMIRY2 fragment was replaced by a fragment containing the coding region of the preprothaumatin gene from *T. danielli* lacking any Pol II promoter sequences.

Plasmid pMIRY4-GC25 contains the coding region of the cytochrome P-450e gene from rat liver (Scholte *et al.*, 1985) flanked by the leader sequence and transcription termination sequence of the yeast L25 ribosomal protein gene (Leer, 1985). The chimeric gene was placed under the control of the yeast *GAL10* promoter (Guarente *et al.*, 1982). Plasmid pMIRY4-GT25 is similar to pMIRY4-GC25 except that it contains the preprothaumatin coding region instead of that from cytochrome P-450e. The construction of both pMIRY4-GC25 and pMIRY4-GT25 is described in Lopes (1990).

Plasmid pMIRY1 (Figure 2) was constructed as follows: (1) the 725 bp *Sma*I-*Hin*dIII rDNA fragment (R1, panel A) was cloned into the polylinker of pUC9 (Vieira and Messing, 1982), resulting in plasmid pUC9-R; (2) the 115 bp *Eco*RV-*Hin*dIII fragment of pUC9-R was substituted by a 55 bp synthetic oligonucleotide containing the following restriction sites: *Nru*I, *Bam*HI, *Xba*I, *Acc*I, *Sal*I, *Hin*cII, *Hin*dIII, *Pst*I, *Sph*I and *Stu*I. This oligonucleotide is flanked by the *Eco*RV site and the 5'-overhanging sequence from the *Hin*dIII site (TCGA), preceded by a CG base pair instead of the correct AT base pair (panel B). In this way, upon ligation, the *Hin*dIII site was not restored, leaving a unique *Hin*dIII site within the inserted oligonucleotide. This sub-clone was called pUC-RO; (3) the 850 bp *Sph*I-*Sna*BI rDNA fragment (R2, panel A) was inserted into pUC9-RO between the *Sph*I and *Stu*I site of the polylinker, giving plasmid pUC9-ROR; (4) finally, the 1·8 kb *Eco*RI-*Xba*I fragment containing the *LEU2d* gene was isolated from sub-clone pUC9-LEU2d and ligated into pUC9-ROR between the *Bam*HI and *Xba*I sites with the aid of a *Bam*HI-*Eco*RI adapter that contains an *Xho*I recognition site, resulting in plasmid pMIRY1.

Plasmids pMIRY1-2, pMIRY1-TOP and pMIRY1-Ta (Tna) are derivatives of pMIRY1 (see Figure 3). For the construction of pMIRY1-2, we have inserted the 3·4 kb *Sph*I-*Bgl*II fragment from the *S. cerevisiae* rDNA unit between the *Sph*I and the *Bam*HI site flanking the right polylinker of pMIRY1 (see Figure 2). In the case of pMIRY1-TOP, the 250 bp *Stu*I-*Eco*RI rDNA fragment (see Figure 3) was first subcloned into pIC20H (Marsh

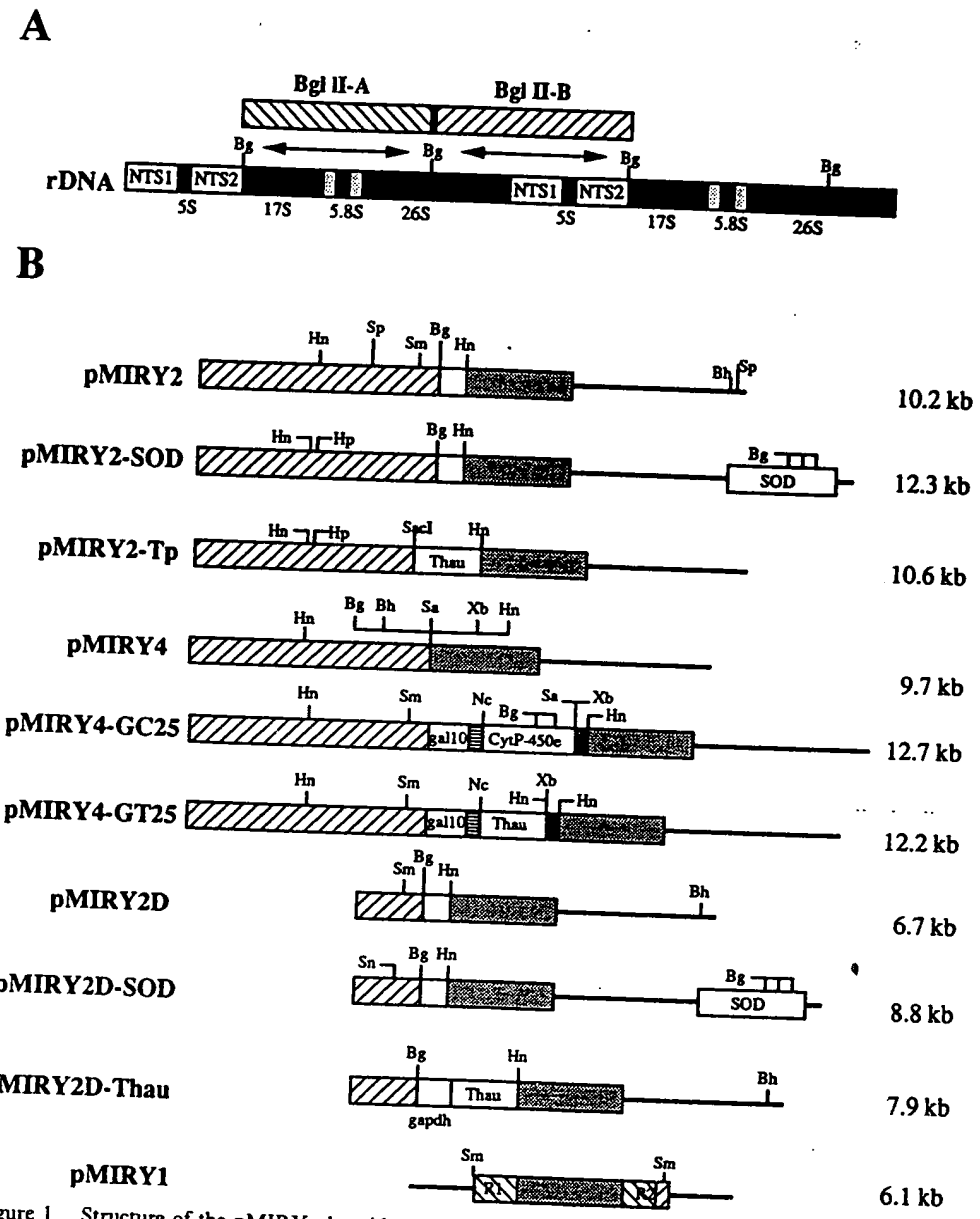

Figure 1. Structure of the pMIRY plasmids. Panel A shows the genetic organization of an rDNA unit from *S. cerevisiae*. The *Bgl*II-A and *Bgl*II-B fragments are indicated. Panel B depicts the structure of the various plasmids described in this paper. The shaded box represents the *LEU2d* selection marker and the black line the pBR322 or pUC9 sequences. The rDNA fragments are indicated as in panel A. The empty box represents the 450 bp *Spirodela olighoriza* sequence present in the original pARES6 plasmid (Kempers-Veenstra *et al.*, 1984). Cloned genes are indicated as follows: *SOD*: yeast Mn^{2+} superoxide dismutase; *CytP-450e*: rat cytochrome P-450e; *Thau*: preprothaumatin from *T. danielli*. Restriction sites are indicated as follows: Bg, *Bgl*II; Bh, *Bam*HI; Hp, *Hpa*I; Hi; *Hin*dIII; Nc, *Nco*I; Sc, *Sac*I; Sm, *Sma*I; Sa, *Sal*I; Sn, *Sna*BI; Sp, *Sph*I; Xh, *Xho*I. gal10 indicates the *GAL10* promoter region and gapdh the *GAPDH* promoter region. Finally, the black box represents the termination region of the yeast L25 ribosomal protein gene and the horizontally striped box the leader sequence of the same gene. Details of the construction are described in Materials and Methods.

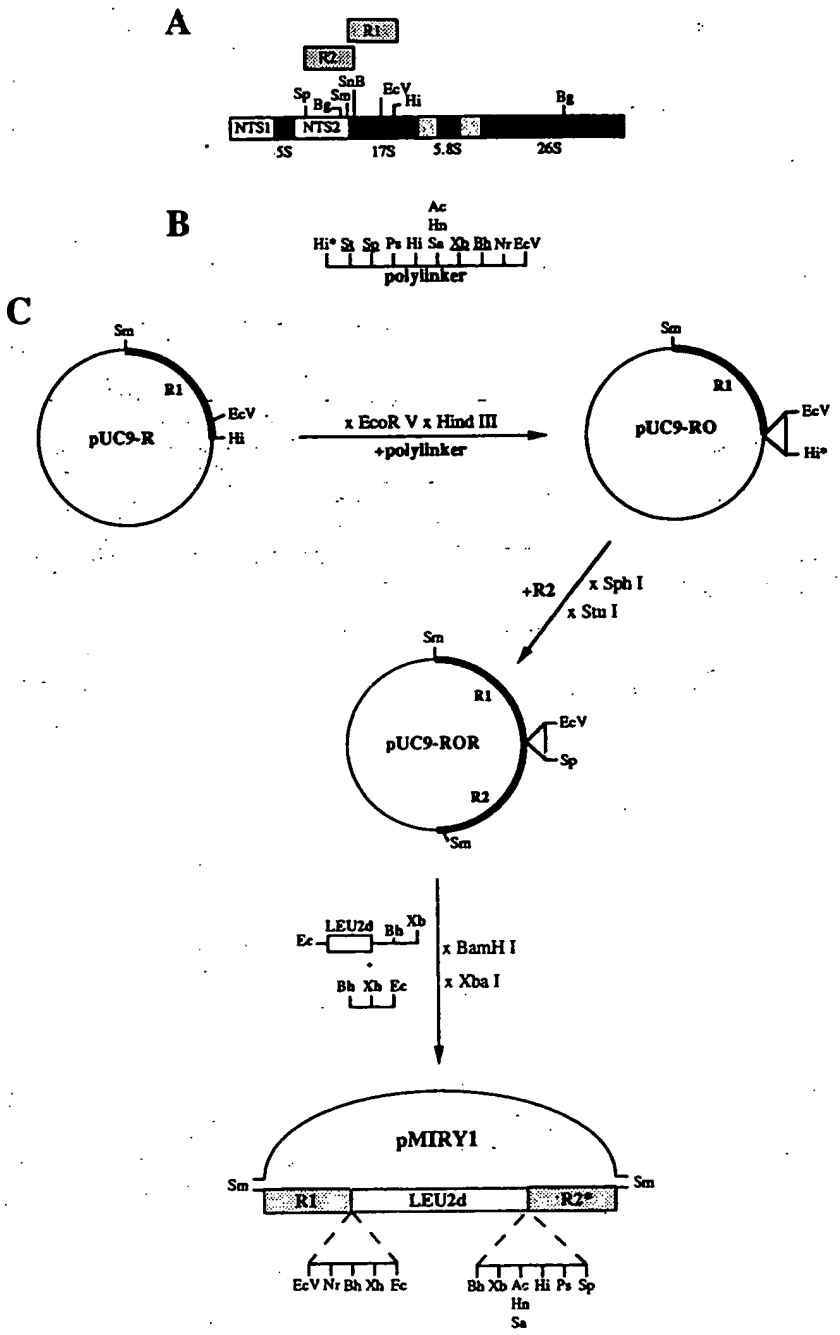

Figure 2. Construction of plasmid pMIRY1. Panel A shows the genetic organization of a single rDNA unit of the yeast *S. cerevisiae* in order to illustrate the rDNA fragments used in the construction of pMIRY1, indicated as R1 and R2. Panel B shows the synthetic polylinker used in the construction of pMIRY1. Panel C outlines the construction. The thick black lines indicate the rDNA fragments R1 and R2. R2* is identical to R2 except for the absence of the small *Sma*I-*Sna*BI fragment. The empty box represents the *LEU2d* gene. Restriction sites are indicated as follows: Ac, *Acc*I; Bh, *Bam*HI; Ec, *Eco*RI; EcV, *Eco*RV; Hi, *Hin*dIII; Hn, *Hin*dII; Nr, *Nru*I; Ps, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; SnB, *Sna*BI; Sp, *Sph*I; Xb, *Xba*I; Xh, *Xho*I.

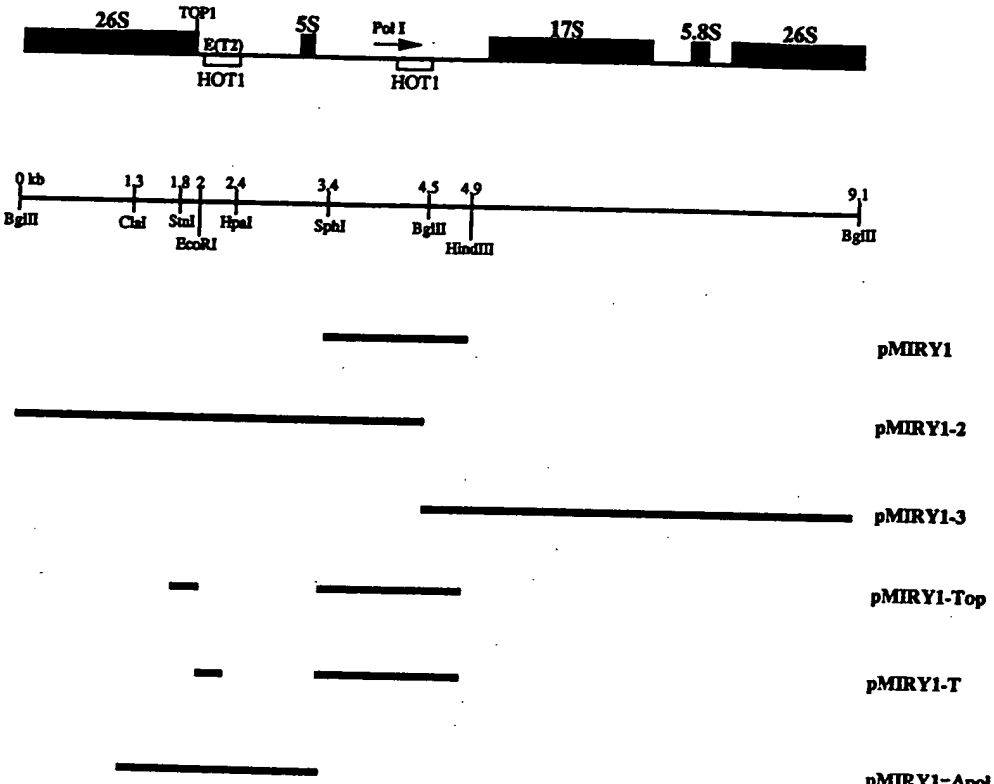

Figure 3. The rDNA fragments cloned in the different pMIRY1 plasmids. At the top, we show the genetic organization of an rDNA unit from *S. cerevisiae*. We have indicated the position of the following regulatory sequences: E: Pol I transcription enhancer; T2: Pol I transcription terminator; HOT1: hot-spot for recombination; TOP1: topoisomerase I binding site. Below and correctly aligned we show as dark lines the various rDNA fragments cloned in each pMIRY1 plasmid.

et al., 1984), afterwards reisolated as a *Xba*I-*Hin*dIII fragment and subsequently introduced between the *Xba*I and *Hin*dIII sites in the right polylinker of pMIRY1 (see Figure 2). Similarly, in the construction of pMIRY1-Ta (Tna), the 200 bp *Eco*RI-*Hpa*I fragment from the rDNA unit was first subcloned in pIC20H, afterwards reisolated as a *Sal*I-*Xho*I fragment and ligated to the *Xho*I linearized pMIRY1. pMIRY1-Ta is the plasmid containing the Pol I transcription-terminating region (van der Sande *et al.*, 1989) in the normal orientation, whereas in pMIRY1-Tna this sequence is in the reverse orientation.

Plasmids pMIRY1-3 and pMIRY1-Δpol were both constructed in a similar manner as pMIRY1 (see Figure 2). In the case of pMIRY1-3 we fused 2·9 kb *Bgl*II-*Hpa*I and the 1·6 kb *Bgl*II-*Sph*I fragments from the rDNA unit (see Figure 3) in pIC20H. Plasmid pMIRY1-3 was completed when the deficient *LEU2* gene was inserted in the *Bgl*II site of the polylinker present between the two rDNA stretches. For the construction of pMIRY1-Δpol we joined the 1·1 kb *Hpa*I-*Sph*I and the 1 kb *Hpa*I-*Cla*I rDNA fragments (see Figure 3) in pIC20. The construction of pMIRY1-Δpol was completed with the insertion of the deficient *LEU2* gene into the polylinker between the two rDNA portions.

Determination of plasmid copy number

Total DNA was extracted from cells of each transformant and digested with either *Bgl*II (in the cases of pMIRY2, pMIRY2D, pMIRY3, pMIRY4-GC25 and pMIRY2/2D-SOD transformants) or with *Hin*dIII (in the case of pMIRY2-Tp and pMIRY4-GT25). *Bgl*II digestion of the genomic ribosomal DNA produces two 4·5 kb fragments, while *Hin*dIII digestion produces a 6·4 kb and a 2·7 kb fragment. The copy number of the plasmid was determined by electrophoresis

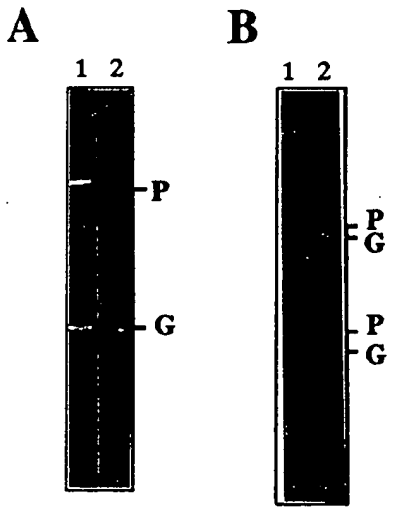

Figure 4. Mitotic stability of transformants containing a pMIRY2-derived expression vector. Total DNA was isolated from pMIRY4-GC25 (panel A) and pMIRY4-GT25 (panel B) transformants at the start of the experiment (lanes 1) and after growth for about 70 generations on non-selective medium containing galactose (lanes 2). The copy numbers were determined as described in Materials and Methods. DNA from pMIRY4-GC25 transformants was digested with *Bgl*II, DNA from pMIRY4-GT25 transformants with *Hin*dIII. In the latter case genomic rDNA gives rise to a 6·4 kb and a 2·7 kb fragment while the plasmid is cleaved into a 7·4 kb and a 4·8 kb fragment. Plasmid and genomic rDNA are indicated by P and G respectively.

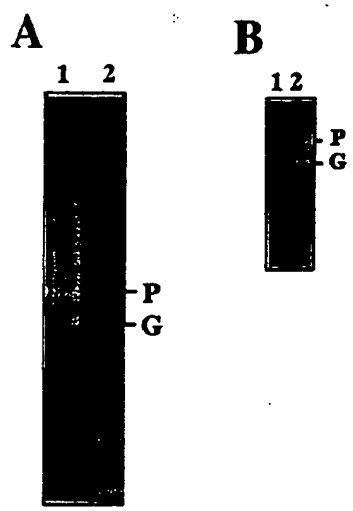

Figure 5. Comparison of stability of pMIRY2-SOD (panel A) and pMIRY2D-SOD (panel B) transformants. Total DNA was isolated from the respective transformants at the start of the experiment (lane 1) and after growth for about 70 generations (lane 2) in non-selective medium. Copy numbers were determined as described in Materials and Methods after digestion of DNA with *Bgl*II. Plasmid and genomic rDNA bands are indicated by P and G respectively. pMIRY2-SOD gives rise to 5·6 and 5·8 kb fragments that are not separated by the gel system used. The smaller fragments produced by *Bgl*II digestion of either plasmid were not detectable.

of the digest on an 0·8% agarose gel and visual comparison of the intensities of the plasmid and the rDNA after staining with EtBr, taking into account the relative lengths of the various fragments.

Determination of the stability of the yeast transformants in batch culture

High-copy-number transformants obtained with each of the pMIRY plasmids were cultured in 20 ml medium containing 0·67% (w/v) yeast nitrogen base (with amino acids) and 2% (w/v) of either glucose or galactose, with shaking at 30°C. When the cultures reached an OD_{550nm} between 2 and 3 they were diluted to an OD_{550nm} of 0·1 to maintain exponential growth. Cell samples were collected after 6–7. 30–35 and 60–70 generations. Total DNA was isolated from these cells (which are all plasmid$^+$) and used for determining the plasmid copy number. In cases where the inserted MIRY-plasmids were not fully maintained after prolonged growth (Figures 4 and 5), the loss of copies was actually completed after about 30 generations. The remaining copies were stably kept.

RESULTS AND DISCUSSION

Influence of gene expression on the mitotic stability of pMIRY-type vectors

We have previously shown that insertion of a (foreign) gene into pMIRY2 does not prevent the high-copy-number integration into the rDNA locus of *S. cerevisiae* (Lopes *et al.*, 1989). However, as demonstrated in Figure 4, the mitotic stability of pMIRY2 vectors carrying such a gene is decreased significantly. Yeast cells transformed with either pMIRY4-GC25, which contains the rat liver cytochrome P450-e cDNA (Scholte *et al.*, 1985), or pMIRY4-GT25, carrying the cDNA for preprothaumatin from *T. danielli* (Edens *et al.*, 1984), both under control of the *GAL10* promoter, lose some 80% of their vector copies over a period of 70 generations of growth in non-selective medium containing galactose as the sole carbon source.

One explanation for the low mitotic stability of the pMIRY4-GC25 and -GT25 plasmids might be

the (long-term) cytotoxicity of the foreign protein. Alternatively, the high copy number of these plasmids might lead to a selective disadvantage because of the extra burden of replication and/or transcription of the numerous copies of the foreign gene. Both factors are known to affect the stability of YEp-type vectors (DiBiasio and Sardonni, 1986). In order to establish whether expression of the inserted gene has a negative effect on plasmid stability, we cultured pMIRY4-GC25 and -GT25 transformants in medium containing glucose instead of galactose as the carbon source in order to shut off transcription of the foreign gene. Even though no expression of either cytochrome P450-e or preprothaumatin could be detected in the respective transformants using ELISA or Western blotting, we found no improvement in plasmid stability under these conditions (data not shown). Thus, the observed instability of the pMIRY2-derived expression vectors is not a direct consequence of over-expression of the heterologous gene and/or toxicity of the protein product.

One might, however, still argue that the large number of *GAL10* promoters present in the transformants acts as a drain for transcription factor(s) required for expression of cellular genes and, consequently, high-copy-number transformants are put at a disadvantage. Gal4p, the major factor interacting with the *GAL10* promoter, is known to bind to the *GAL* UAS even in the absence of galactose (Johnston, 1987). To clarify this point we have constructed plasmid pMIRY2-Tp, containing the preprothaumatin gene without any yeast RNA polymerase II promoter sequences (Figure 1). pMIRY2-Tp transformants, however, proved to be highly unstable (data not shown), refuting the idea that the instability of the pMIRY2-derived expression vectors is due to a depletion of transcription factors by the *GAL10* promoters.

Influence of plasmid size on the mitotic stability of pMIRY-type vectors

Insertion of either the cytochrome P450-e or the preprothaumatin cDNA into pMIRY2 lengthens the plasmid by 2–2·5 kb. In order to establish whether this increase in size might be responsible for the loss of mitotic stability, we cloned either the preprothaumatin cDNA, under control of the yeast *GAPDH* promoter, or the homologous Mn^{2+} superoxide dismutase (*SOD*) gene, under control of its own promoter, into pMIRY2D (Figure 1). This plasmid is a derivative of pMIRY2 obtained by removing approximately two-thirds of the rDNA fragment (up to the *Sph*I site). Consequently the pMIRY2D-Thau and pMIRY2D-SOD plasmids are 3–4 kb shorter than pMIRY4-GT25 and pMIRY2-SOD, respectively (Figure 1). Figure 5 shows a representative copy number analysis of both a pMIRY2-SOD (panel A) and a pMIRY2D-SOD (panel B) transformant before and after long-term growth in non-selective medium. The first plasmid (12·3 kb) shows the same loss of vector copies as the pMIRY4-GC25 and -GT25 transformants discussed above. Reducing the size of the vector to 8·8 kb, however, results in a dramatic improvement of mitotic stability: the copy number of pMIRY2D-SOD transformants (100–150) does not change significantly over a period of about 70 generations of growth in non-selective medium. The same result was obtained for pMIRY2D-Thau (data not shown), indicating that indeed plasmid size is a crucial factor in determining mitotic stability of pMIRY-type vectors. Apparently, there is a relatively sharp transition from a mitotically stable to an unstable vector somewhere around a size of approximately 9–10 kb, i.e. about the length of an rDNA unit. Even lengthening pMIRY2 by 0·4 kb, as in the case of pMIRY2-Tp (see Figure 1) results in reduction of stability (see above). In conclusion, pMIRY-type vectors are only stably maintained in the rDNA cluster if their size is smaller than or at most equal to the size of the rDNA unit (9·1 kb). In addition, the stability of these vectors is determined by the presence of certain rDNA elements (see next section).

Apart from the effect on mitotic stability, plasmid length also seems to influence the initial copy number of pMIRY-type vectors. This is illustrated by the data in Figure 6 which show the initial copy number analysis of two independently obtained pMIRY2D transformants. Clearly the 6·7 kb plasmid and 4·5 kb genomic rDNA bands have approximately the same staining intensity. Since the rDNA band represents 9·1 kb of DNA (the *Bgl*II-A plus the *Bgl*II-B fragment; cf. Figure 1), we conclude that the copy number of pMIRY2D is about 180 (1·3 × 140, the total number of rDNA repeats), some 50% higher than the copy number typically observed for pMIRY2 transformants (Lopes *et al.*, 1989). It seems reasonable to assume that this inverse correlation between plasmid length and copy number reflects a limitation in the amount of extra DNA that can be accommodated by chromosome XII.

Figure 6. Copy number determination of pMIRY2D transformants. Total DNA was digested with *Bgl*II and copy number determined as described in Materials and Methods. The plasmid and genomic rDNA bands are indicated by P and G respectively.

Figure 7. Copy number analysis of cells transformed with pMIRY1 free of bacterial sequences. Yeast cells were transformed with pMIRY1 digested with *Sma*I (cf. Figure 1). Total DNA from two independently obtained transformants was digested with *Bgl*II and copy number was determined as described in Materials and Methods. The plasmid and genomic rDNA bands are indicated by P and G respectively.

Influence of the nature of the rDNA present in the pMIRY-type vector on its mitotic stability

The observation that copy number can be increased and stability improved by reducing the size of a pMIRY-type plasmid, prompted us to construct a vector even smaller than pMIRY2D. The result is plasmid pMIRY1 (see Figure 1), which upon cutting with *Sma*I gives rise to an integrating fragment of 3·4 kb consisting of two rDNA sequences separated by the *LEU2d* selection marker. The presence in *cis* of the latter is absolutely required for high-copy integration (Lopes *et al.*, 1991). This plasmid also has the additional advantage that no bacterial sequences are introduced into the yeast transformants. It has been reported previously (Lusky and Botchian, 1981) that bacterial sequences are potentially toxic to other organisms and therefore may decrease vector stability in yeast (Awane *et al.*, 1992). Besides, bacterial DNA is not acceptable in a so-called 'food-grade-vector', a status which is an advantage to gain clearance for use in the commercial biotechnological production of proteins.

In Figure 7 we show the copy number analysis of two independently obtained pMIRY1 transformants. From the similar staining intensities of the 4·5 kb (double) rDNA band and the 3·4 kb plasmid band we conclude that pMIRY1 is present in about 400 copies per cell, to our knowledge the highest value ever obtained so far in yeast. Unexpectedly, however, the transformants rapidly lost most of their vector copies when grown under non-selective conditions (not shown). Thus plasmid length is definitely not the sole factor determining mitotic stability. Another cause for the disparity in stability of the various pMIRY-type vectors might lie in differences between their rDNA portions. It can be postulated that the unique combination of properties of the pMIRY vectors, i.e. a high copy number and stable maintenance, is related to the fact that they are integrated in the rDNA locus.

The *S. cerevisiae* rDNA unit contains three elements that appear to play important roles in the maintenance of the approximately 140 homogeneous, tandem repeats of this unit. Two of these fragments (HOT1; see Figure 3) together stimulate recombination in a transcription-dependent manner (Keil and Roeder, 1984; Voelkel-Meiman *et al.*, 1987; Stewart and Roeder, 1989), possibly as a consequence of supercoiling induced by unwinding of the rDNA by the transcription complex (Voelkel-Meiman *et al.*, 1987; Liu and Wang, 1987). The resulting torsional stress would, however, be relieved periodically by the action of topoisomerases binding to the TOP1 site close to the 3′-end of the 26S rRNA (Figure 3; Liu and Wang, 1987; Voelkel-Meiman *et al.*, 1987). In accordance with this hypothesis, deleterious mutations in the genes encoding topoisomerase I and II were found to cause an increase in rDNA recombination in yeast (Christman *et al.*, 1988; Kim and Wang, 1989). Thus, maintenance of the size of the rDNA cluster and the homogeneity of the reiterated units would be due to an equilibrium between the stimulatory activity of the HOT1 sites on recombination and its repression via the TOP1

Table 1. Mitotic stability of pMIRY1-derived plasmids.

Plasmid	Prom	Term	TOP1 b.s.	Stability
pMIRY1	+	−	−	−
pMIRY1-2	+	+	+	+
pMIRY1-3	−	−	−	+
pMIRY1-Top	+	−	+	+
pMIRY1-Ta	+	→	−	+
pMIRY1-Tna	+	←	−	−
pMIRY1-ΔP	−	+	+	+

Yeast cells were transformed with pMIRY1 derivatives containing different rDNA fragments as indicated in Figure 3. Regulatory sequences are: Prom, RNA polymerase I promoter; Term, RNA polymerase I terminator (van der Sande *et al.*, 1989); TOP1 b.s., binding site for topoisomerase I. Transformants containing 300–400 copies of the integrated plasmid were grown in non-selective medium and the copy number of the plasmid was determined periodically. Plasmid stability: +, less than 20% loss; −, more than 20% loss over 40–60 generations.

site. The same sites may be required for the stable maintenance of the pMIRY-type vectors.

In order to ascertain the validity of this hypothesis we constructed a series of plasmids containing various combinations of the Pol I transcription initiation and termination (T2) region, HOT1 and TOP1 sites (Figure 3; note that the two HOT1 sites correspond to sequences in the transcription initiation and transcriptional enhancer/terminator regions of the rDNA respectively). For each of these plasmids we selected several transformants carrying 300–400 integrated copies and periodically determined the copy number during growth for 40–60 generations on non-selective medium. The results, summarized in Table 1, show that the stability of the pMIRY1 vector is dramatically improved by either including the Pol I transcription-terminating region (van der Sande *et al.*, 1989) in the vector (pMIRY1-Ta), or by excluding the Pol I transcription initiation region from it (pMIRY1-3; pMIRY1-Δpol). A similar stabilization can also be effected by inserting the TOP1 site into pMIRY1 (pMIRY1-Top). These data indeed support our hypothesis that the regulatory elements responsible for the stability of the rDNA units are also required to stabilize the pMIRY-type integrated vectors. It should be noted, however, that the putative origins of replication present within the NTS2 are not required for stable maintenance, as can be inferred from the stability of pMIRY1-3 (see Figure 3) in which these elements are absent. These findings have, therefore, to be taken into account in the design of a pMIRY vector to be used for industrial purposes.

An interesting observation is the difference in the mitotic stability between plasmid pMIRY2D and pMIRY1. Contrary to pMIRY1, pMIRY2D was found to be stable in spite of the presence of the entire Pol I promoter region which extends from position −155 to +27 relative to the transcription start site (Musters *et al.*, 1989). Unlike pMIRY2D, pMIRY1 contains almost the complete intergenic spacer including the binding sites for protein Reb1p (Kulkens *et al.*, 1989; Morrow *et al.*, 1989), which seems to be involved in bringing the promoter into optimal spatial configuration with respect to the transcription initiation site (Kulkens *et al.*, 1992). In addition, although the HOT1 element overlapping the Pol I promoter is present in both plasmids, in the case of pMIRY2D this element is not in its natural context, which might influence its action, as implied by Voelkel-Meiman and Roeder (1990) and Ponticelli and Smith (1992). These differences between the rDNA fragments present in pMIRY2D and pMIRY1 might account for the difference in the stability of the two plasmids.

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§ 2144.09 for examples of reasoning supporting obviousness rejections.

When the motivation to combine the teachings of the references is not immediately apparent, it is the duty of the examiner to explain why the combination of the teachings is proper. *Ex parte Skinner*, 2 USPQ2d 1788 (Bd. Pat. App. & Inter. 1986). A statement of a rejection that includes a large number of rejections must explain with reasonable specificity at least one rejection, otherwise the examiner procedurally fails to establish a *prima facie* case of obviousness. *Ex parte Blanc*, 13 USPQ2d 1383 (Bd. Pat. App. & Inter. 1989) (Rejection based on nine references which included at least 40 prior art rejections without explaining any one rejection with reasonable specificity was reversed as procedurally failing to establish a *prima facie* case of obviousness.).

If the examiner determines there is factual support for rejecting the claimed invention under 35 U.S.C. 103, the examiner must then consider any evidence supporting the patentability of the claimed invention, such as any evidence in the specification or any other evidence submitted by the applicant. The ultimate determination of patentability is based on the entire record, by a preponderance of evidence, with due consideration to the persuasiveness of any arguments and any secondary evidence. *In re Oetiker*, 977 F.2d 1443, 24 USPQ2d 1443 (Fed. Cir. 1992). The legal standard of "a preponderance of evidence" requires the evidence to be more convincing than the evidence which is offered in opposition to it. With regard to rejections under 35 U.S.C. 103, the examiner must provide evidence which as a whole shows that the legal determination sought to be proved (i.e., the reference teachings establish a *prima facie* case of obviousness) is more probable than not.

When an applicant submits evidence, whether in the specification as originally filed or in reply to a rejection, the examiner must reconsider the patentability of the claimed invention. The decision on patentability must be made based upon consideration of all the evidence, including the evidence submitted by the examiner and the evidence submitted by the applicant. A decision to make or maintain a rejection in the face of all the evidence must show that it was based on the totality of the evidence. Facts established by rebuttal evidence must be evaluated along with the facts on which the conclusion of obviousness was reached, not against the conclusion itself. *In re Eli Lilly & Co.*, 902 F.2d 943, 14 USPQ2d 1741 (Fed. Cir. 1990).

See *In re Piasecki*, 745 F.2d 1468, 223 USPQ 785 (Fed. Cir. 1984) for a discussion of the proper roles of the examiner's *prima facie* case and applicant's rebuttal evidence in the final determination of obviousness. See MPEP § 706.02(j) for a discussion of the proper contents of a rejection under 35 U.S.C. 103.

2143 Basic Requirements of a *Prima Facie* Case of Obviousness

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

2143.01 Suggestion or Motivation To Modify the References [R-2]

THE PRIOR ART MUST SUGGEST THE DESIRABILITY OF THE CLAIMED INVENTION

"There are three possible sources for a motivation to combine references: the nature of the problem to be solved, the teachings of the prior art, and the knowledge of persons of ordinary skill in the art." *In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998) (The combination of the references taught every element of the claimed invention, however without a motivation to combine, a rejection based on a *prima facie* case of obvious was held improper.). The level of skill in the art cannot be relied upon to provide the suggestion to combine references. *Al-Site Corp. v. VSI Int'l Inc.*, 174 F.3d 1308, 50 USPQ2d 1161 (Fed. Cir. 1999).

"In determining the propriety of the Patent Office case for obviousness in the first instance, it is necessary to ascertain whether or not the reference teachings would appear to be sufficient for one of ordinary skill in the relevant art having the reference before him to make the proposed substitution, combination, or other modification." *In re Linter*, 458 F.2d 1013, 1016, 173 USPQ 560, 562 (CCPA 1972).

Obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art. "The test for an implicit showing is what the combined teachings, knowledge of one of ordinary skill in the art, and the nature of the problem to be solved as a whole would have suggested to those of ordinary skill in the art." *In re Kotzab,* 217 F.3d 1365, 1370, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000). See also *In re Lee,* 277 F.3d 1338, 1342-44, 61 USPQ2d 1430, 1433-34 (Fed. Cir. 2002) (discussing the importance of relying on objective evidence and making specific factual findings with respect to the motivation to combine references); *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988); *In re Jones*, 958 F.2d 347, 21 USPQ2d 1941 (Fed. Cir. 1992).

>In *Ruiz v. A.B. Chance Co.*, 357 F.3d 1270, 69 USPQ2d 1686 (Fed. Cir. 2004), the patent claimed underpinning a slumping building foundation using a screw anchor attached to the foundation by a metal bracket. One prior art reference taught a screw anchor with a concrete bracket, and a second prior art reference disclosed a pier anchor with a metal bracket. The court found motivation to combine the references to arrive at the claimed invention in the "nature of the problem to be solved" because each reference was directed "to precisely the same problem of underpinning slumping foundations." *Id.* at 1276, 69 USPQ2d at 1690. The court also *rejected* the notion that "an express written motivation to combine must appear in prior art references...." *Id.* at 1276, 69 USPQ2d at 1690.<

In *In re Kotzab*, the claims were drawn to an injection molding method using a single temperature sensor to control a plurality of flow control valves. The primary reference disclosed a multizone device having multiple sensors, each of which controlled an associated flow control valve, and also taught that one *system* may be used to control a number of valves. The court found that there was insufficient evidence to show that one *system* was the same as one *sensor.* While the control of multiple valves by a single sensor rather than by multiple sensors was a "technologically simple concept," there was no finding "as to the specific understanding or principle within the knowledge of the skilled artisan" that would have provided the motivation to use a single sensor as the system to control more than one valve. 217 F.3d at 1371, 55 USPQ2d at 1318.

In *In re Fine*, the claims were directed to a system for detecting and measuring minute quantities on nitrogen compounds comprising a gas chromatograph, a converter which converts nitrogen compounds into nitric oxide by combustion, and a nitric oxide detector. The primary reference disclosed a system for monitoring sulfur compounds comprising a chromatograph, combustion means, and a detector, and the secondary reference taught nitric oxide detectors. The examiner and Board asserted that it would have been within the skill of the art to substitute one type of detector for another in the system of the primary reference, however the court found there was no support or explanation of this conclusion and reversed.

In *In re Jones*, the claimed invention was the 2-(2¢-aminoethoxy) ethanol salt of dicamba, a compound with herbicidal activity. The primary reference disclosed *inter alia* the substituted ammonium salts of dicamba as herbicides, however the reference did not specifically teach the claimed salt. Secondary references teaching the amine portion of the salt were directed to shampoo additives and a byproduct of the production of morpholine. The court found there was no suggestion to combine these references to arrive at the claimed invention.

WHERE THE TEACHINGS OF THE PRIOR ART CONFLICT, THE EXAMINER MUST WEIGH THE SUGGESTIVE POWER OF EACH REFERENCE

The test for obviousness is what the combined teachings of the references would have suggested to one of ordinary skill in the art, and all teachings in the prior art must be considered to the extent that they are in analogous arts. Where the teachings of two or more

prior art references conflict, the examiner must weigh the power of each reference to suggest solutions to one of ordinary skill in the art, considering the degree to which one reference might accurately discredit another. *In re Young*, 927 F.2d 588, 18 USPQ2d 1089 (Fed. Cir. 1991) (Prior art patent to Carlisle disclosed controlling and minimizing bubble oscillation for chemical explosives used in marine seismic exploration by spacing seismic sources close enough to allow the bubbles to intersect before reaching their maximum radius so the secondary pressure pulse was reduced. An article published several years later by Knudsen opined that the Carlisle technique does not yield appreciable improvement in bubble oscillation suppression. However, the article did not test the Carlisle technique under comparable conditions because Knudsen did not use Carlisle's spacing or seismic source. Furthermore, where the Knudsen model most closely approximated the patent technique there was a 30% reduction of the secondary pressure pulse. On these facts, the court found that the Knudsen article would not have deterred one of ordinary skill in the art from using the Carlisle patent teachings.).

FACT THAT REFERENCES CAN BE COMBINED OR MODIFIED IS NOT SUFFICIENT TO ESTABLISH *PRIMA FACIE* OBVIOUSNESS

The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990) (Claims were directed to an apparatus for producing an aerated cementitious composition by drawing air into the cementitious composition by driving the output pump at a capacity greater than the feed rate. The prior art reference taught that the feed means can be run at a variable speed, however the court found that this does not require that the output pump be run at the claimed speed so that air is drawn into the mixing chamber and is entrained in the ingredients during operation. Although a prior art device "may be capable of being modified to run the way the apparatus is claimed, there must be a suggestion or motivation in the reference to do so." 916 F.2d at 682, 16 USPQ2d at 1432.). See also *In re Fritch*, 972 F.2d 1260, 23 USPQ2d 1780 (Fed. Cir. 1992) (flexible landscape edging device which is conformable to a ground surface of varying slope not suggested by combination of prior art references).

FACT THAT THE CLAIMED INVENTION IS WITHIN THE CAPABILITIES OF ONE OF ORDINARY SKILL IN THE ART IS NOT SUFFICIENT BY ITSELF TO ESTABLISH *PRIMA FACIE* OBVIOUSNESS

A statement that modifications of the prior art to meet the claimed invention would have been " 'well within the ordinary skill of the art at the time the claimed invention was made' " because the references relied upon teach that all aspects of the claimed invention were individually known in the art is not sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teachings of the references. *Ex parte Levengood*, 28 USPQ2d 1300 (Bd. Pat. App. & Inter. 1993). See also *In re Kotzab*, 217 F.3d 1365, 1371, 55 USPQ2d 1313, 1318 (Fed. Cir. 2000) (Court reversed obviousness rejection involving technologically simple concept because there was no finding as to the principle or specific understanding within the knowledge of a skilled artisan that would have motivated the skilled artisan to make the claimed invention); *Al-Site Corp. v. VSI Int'l Inc.*, 174 F.3d 1308, 50 USPQ2d 1161 (Fed. Cir. 1999) (The level of skill in the art cannot be relied upon to provide the suggestion to combine references.).

THE PROPOSED MODIFICATION CANNOT RENDER THE PRIOR ART UNSATISFACTORY FOR ITS INTENDED PURPOSE

If proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984) (Claimed device was a blood filter assembly for use during medical procedures wherein both the inlet and outlet for the blood were located at the bottom end of the filter assembly, and wherein a gas vent was present at the top of the filter assembly. The prior art reference taught a liquid strainer for removing dirt and water from gasoline and other light oils wherein the inlet and outlet were at the top of the device, and wherein a pet-cock (stopcock) was located at the bottom of the device for periodically removing the

collected dirt and water. The reference further taught that the separation is assisted by gravity. The Board concluded the claims were *prima facie* obvious, reasoning that it would have been obvious to turn the reference device upside down. The court reversed, finding that if the prior art device was turned upside down it would be inoperable for its intended purpose because the gasoline to be filtered would be trapped at the top, the water and heavier oils sought to be separated would flow out of the outlet instead of the purified gasoline, and the screen would become clogged.).

"Although statements limiting the function or capability of a prior art device require fair consideration, simplicity of the prior art is rarely a characteristic that weighs against obviousness of a more complicated device with added function." *In re Dance*, 160 F.3d 1339, 1344, 48 USPQ2d 1635, 1638 (Fed. Cir. 1998) (Court held that claimed catheter for removing obstruction in blood vessels would have been obvious in view of a first reference which taught all of the claimed elements except for a "means for recovering fluid and debris" in combination with a second reference describing a catheter including that means. The court agreed that the first reference, which stressed simplicity of structure and taught emulsification of the debris, did not teach away from the addition of a channel for the recovery of the debris.).

THE PROPOSED MODIFICATION CANNOT CHANGE THE PRINCIPLE OF OPERATION OF A REFERENCE

If the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the references are not sufficient to render the claims *prima facie* obvious. *In re Ratti*, 270 F.2d 810, 123 USPQ 349 (CCPA 1959) (Claims were directed to an oil seal comprising a bore engaging portion with outwardly biased resilient spring fingers inserted in a resilient sealing member. The primary reference relied upon in a rejection based on a combination of references disclosed an oil seal wherein the bore engaging portion was reinforced by a cylindrical sheet metal casing. Patentee taught the device required rigidity for operation, whereas the claimed invention required resiliency. The court reversed the rejection holding the "suggested combination of references would require a substantial reconstruction and redesign of the elements shown in [the primary reference] as well as a change in the basic principle under which the [primary reference] construction was designed to operate." 270 F.2d at 813, 123 USPQ at 352.).

2143.02 Reasonable Expectation of Success Is Required

OBVIOUSNESS REQUIRES ONLY A REASONABLE EXPECTATION OF SUCCESS

The prior art can be modified or combined to reject claims as *prima facie* obvious as long as there is a reasonable expectation of success. *In re Merck & Co., Inc.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986) (Claims directed to a method of treating depression with amitriptyline (or nontoxic salts thereof) were rejected as *prima facie* obvious over prior art disclosures that amitriptyline is a compound known to possess psychotropic properties and that imipramine is a structurally similar psychotropic compound known to possess antidepressive properties, in view of prior art suggesting the aforementioned compounds would be expected to have similar activity because the structural difference between the compounds involves a known bioisosteric replacement and because a research paper comparing the pharmacological properties of these two compounds suggested clinical testing of amitriptyline as an antidepressant. The court sustained the rejection, finding that the teachings of the prior art provide a sufficient basis for a reasonable expectation of success.); *Ex parte Blanc*, 13 USPQ2d 1383 (Bd. Pat. App. & Inter. 1989) (Claims were directed to a process of sterilizing a polyolefinic composition with high-energy radiation in the presence of a phenolic polyester antioxidant to inhibit discoloration or degradation of the polyolefin. Appellant argued that it is unpredictable whether a particular antioxidant will solve the problem of discoloration or degradation. However, the Board found that because the prior art taught that appellant's preferred antioxidant is very efficient and provides better results compared with other prior art antioxidants, there would have been a reasonable expectation of success.).

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FULL TEXT OF CASES (USPQ2D)
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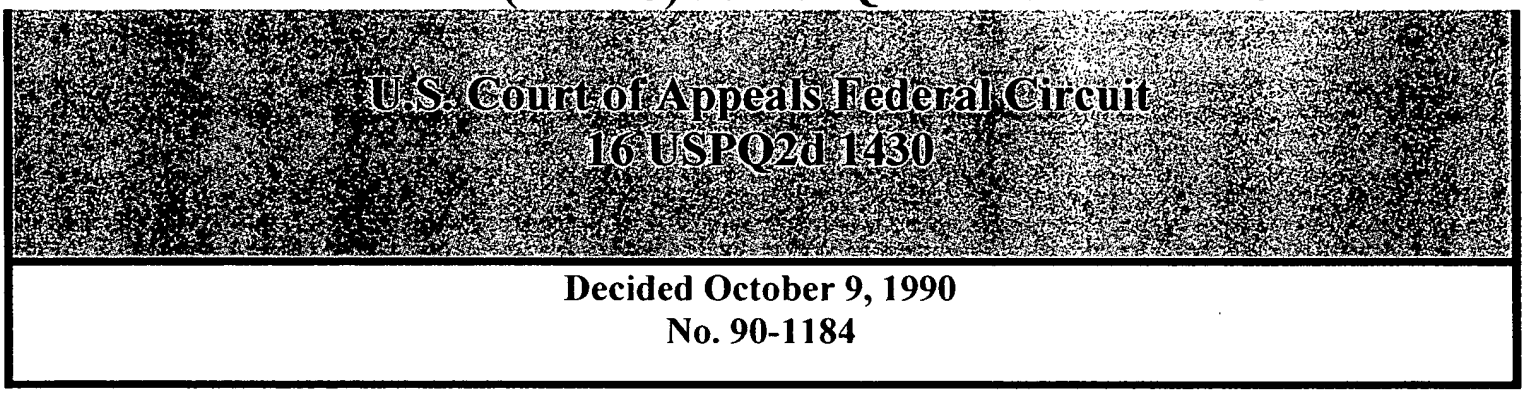
In re Mills (CA FC) 16 USPQ2d 1430 In re Mills



Decided October 9, 1990
No. 90-1184

Headnotes

PATENTS

1. Patentability/Validity - Obviousness - Relevant prior art - Particular inventions (§ 115.0903.03)

Apparatus which produces aerated cementitious composition by driving output pump for its mixing chamber at capacity greater than feed rate of ingredients into mixing chamber, and thereby drawing air into composition, is not obvious in view of prior patent for mixing apparatus, even though device of prior patent provides for regulation of flow rate into mixing chamber, since patent contains no suggestion or motivation for overdriving output pump so as to entrain air in mixed ingredients.

2. Patentability/Validity - Anticipation - In general (§ 115.0701)

Patentability/Validity - Obviousness - Relevant prior art - In general (§ 115.0903.01)

Board of Patent Appeals and Interferences erred by requiring applicant to show that prior art reference lacked functional characteristics of claimed device, since even though such requirement would be proper for rejection based on lack of novelty, it is not pertinent whether prior art device possesses claimed invention's functional characteristics if, as here, application was rejected on basis of obviousness and reference does not describe or

suggest claimed invention's structure.

Case History and Disposition:

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent application of Peter S. Mills, serial no. 891,374, continuation of serial no. 607-805, filed May 4, 1984. From decision upholding examiner's rejection of claims 6-9 and 11-14, applicant appeals. Reversed.

Attorneys:

James C. Wray, McLean, Va, for appellant.

Muriel E. Crawford, assistant solicitor (Fred E. McKelvey, solicitor, with her on brief), for appellee.

Judge:

Before Miller, senior circuit judge, and Mayer and Lourie, circuit judges.

Opinion Text

Opinion By:

Lourie, J.

This appeal is from the November 2, 1989, decision of the United States Patent and Trademark Office Board of Patent Appeals and Interferences (Board), Appeal No. 88-0141, affirming the examiner's rejection, under 35 U.S.C. §103, of claims 6-9 and 11-14 in Mills' application Serial No. 891,374, a continuation of Serial No. 607-805, filed May 4, 1984, entitled "Methods of and Apparatus for Producing Aerated Cementitious Compounds." The remainder of the claims (1-5, 10, and 15) have all been cancelled. We reverse.

I

BACKGROUND

A. *The Invention*

Mills' claimed invention is an apparatus for producing aerated cementitious compositions. Claim 6 is the broadest claim:

6. Apparatus for producing an aerated cementitious composition, comprising
a mixing chamber being open to atmosphere and containing mixing means,
feed means for feeding ingredients comprising cement, foaming agent and liquid to the mixing chamber,
mixing means for mixing ingredients fed to the mixing chamber, pump means for pumping the mixed ingredients to a desired site and having a pump inlet connected to an outlet of the mixing chamber,
drive motor means connected through gearbox means providing a pumping capacity of the pump means greater than the feed rate of the ingredients to the mixing chamber provided by the feed means, such that in operation air is drawn into the mixing chamber, and entrained in the mixed ingredients.
The essence of Mills' invention is the machine's ability to aerate a cementitious composition by driving the output pump at a capacity greater than the feed rate, thereby drawing air into the composition. This aeration produces a composition with substantially lower density than standard cementitious composition mixing ingredients.

B. *The Reference*

The sole reference upon which the Board relied in affirming the examiner's rejection was Mathis et al. U.S. Patent 4,117,547 (Mathis). 1 Mathis discloses a mixing chamber which is open to the atmosphere and which contains a mixing means. Two feed means for feeding ingredients in the mixing chamber are provided. The first feed means may consist of a screw conveyer and the second, a flow metering device such as an adjustable valve. A pump means pumps the mixture from the mixing chamber to a desired site and a drive motor means is connected to mixing means and pump means. A separate motor drives the feed means.
A control system exists to arrest the feed means so as not to overfill the mixing chamber. This system comprises a level detector in the mixing chamber, which signals the feed means to close when the mixing chamber stores the predetermined maximum permissible quantity of material.

C. *The Rejection*

The Board affirmed the examiner's Section 103 rejection of claims 6-9 and 11-14, "finding correspondence in the Mathis reference for all of the subject matter recited in the appellants' claims. ..." With regard to Mills' claim language relating to aerating the mixture, the Board stated: "[i]n our opinion, the differences between claim 6 and the Mathis machine ... lie solely in the functional language of the claim." The Board further found that Mathis teaches the use of separate input and output motors in order to permit the various mixing means and pumps to operate at different rates, and that Mathis "contemplates a situation wherein the rate of the outlet pump would be greater than the inlet pumps...." The Board concluded on this point: "[w]e are of the opinion that the Mathis machine is capable of being operated in such a fashion as to cause [the output] pump 18 to draw air into the mixing chamber 17 so that it is entrained in the mixture."
The Board also agreed with Mills' contention that Mathis is not directed to the problem of producing aerated cementitious material, but noted that Mills is not claiming a method, but an apparatus, and all of Mills' apparatus structure is present in the Mathis machine.

II

DISCUSSION

All of the rejected claims are apparatus claims. The Board found "correspondence in the Mathis reference for all of the subject matter recited in appellants' claims" and that "[t]he Mathis machine discloses all of the structure set forth in claim 1" (a method claim not before us). It asserts that the use of such a mechanism would have been obvious and that the differences between claim 6 and the Mathis machine lie solely in the functional language of

the claim, the preamble merely stating an intended use for the machine. This language suggests a lack of novelty rejection under 35 U.S.C. §102, rather than an obviousness rejection. However, no Section 102 rejection has been made or is before us. What is before us is a rejection for obviousness, and we must decide whether the Board erred in that rejection.

We note first that nonobviousness is a question of law to be determined from the facts. *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1535, 218 USPQ 871, 876 (Fed. Cir. 1983). We review the Board's determination of obviousness, based on the scope and content of the Mathis reference and the differences between the Mathis reference and the Mills claims, for correctness or error. *In re Carleton,* 599 F.2d 1021 1024 n.14, 202 USPQ 165 , 169 n.14 (CCPA 1979).

[1] After reviewing the record, the arguments in the briefs, and the Mathis reference, we conclude that Mathis would not have rendered the claimed invention obvious. The closest Mathis comes to suggesting Mills' claimed apparatus is at column 3, lines 42-47, which states

he rate at which the inlet 2b receives a solid constituent depends on the speed of the feed screw 4. Such speed can be regulated by a prime mover 6 which includes a variable-speed transmission.

This brief reference contains no suggestion of "pump means and the feed means providing a pumping capacity of the pump means greater than the feed rate of ingredients to the mixing chamber provided by the feed means, such that in operation air is drawn into the mixing chamber, and air entrained in the mixed ingredients," as provided for in Mills' claim 6. While Mathis' apparatus may be capable of being modified to run the way Mills' apparatus is claimed, there must be a suggestion or motivation in the reference to do so. *See In re Gordon,* 733 F.2d 900, 902, 221 USPQ 1125, 1127 (Fed. Cir. 1984) ("The mere fact that the prior art could be so modified would not have made the modification obvious unless the prior art suggested the desirability of the modification."). We see no such suggestion. The apparatus claimed by Mills is different from that of Mathis, since the fact that motor 6 of Mathis (the feed means) can be run at a variable speed does not require that motor 20 (connected to the pump) be run at a lesser speed "such that in operation air is drawn into the mixing chamber and air entrained in the mixed ingredients."

[2] The Board found that the difference between the claimed subject matter and the prior art resided solely in functional language and that appellant had to show that the prior art device lacked the functional characteristics of the claimed device, citing *In re Ludtke,* 441 F.2d 660, 169 USPQ 563 (CCPA 1971). *Ludtke,* however, dealt with a rejection for lack of novelty, in which case it was proper to require that a prior art reference cited as anticipating a claimed invention be shown to lack the characteristics of the claimed invention. That proof would in fact negate the assertion that the claimed invention was described in the prior art. We are here, however, facing an obvious

ness issue. It is not pertinent whether the prior art device possesses the functional characteristics of the claimed invention if the reference does not describe or suggest its structure. That is the case here. Given the facts before us, we hold that the Board was in error in affirming the examiner's rejection of claims 6-9 and 11-13 as obvious in view of Mathis, and we therefore *reverse* the Board.

REVERSED

Footnotes

Footnote 1. The examiner rejected the claims at issue under 35 U.S.C. §103 as being unpatentable not only over Mathis but also in view of Gibson et al. U.S. Patent 2,717,770. However, the Board affirmed the examiner's

rejection of claims 6-9 and 11-14 based solely on the Mathis reference. With regard to Gibson the Board stated: We view the teachings of Gibson at best as being merely confirmatory of the fact that aerated mixtures can be produced by machines in which a pump means operates upon a mixing chamber at a greater rate than the ingredients are fed thereunto so that air is drawn into the mixing chamber and entrained in the mixed ingredients. App. 2.

- End of Case -

Intellectual Property Library ISSN 1526-8535

Ex parte Clapp

(BdPatApp&Int)
227 USPQ 972
Opinion dated Feb. 28, 1985
U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences

Headnotes

PATENTS

1. Anticipation -- Combining references (§ 51.205)

To support conclusion that claimed combination is directed to obvious subject matter, references must either expressly or impliedly suggest claimed combination or examiner must present convincing line of reasoning as to why artisan would have found claimed invention to have been obvious in light of references' teachings.

Case History and Disposition:

Application for patent of ThomasR. Clapp, Serial No. 257,162, filed Apr. 24, 1981. From rejection of Claim 9-19, applicant appeals (Appeal No. 553-54). Reversed.

Attorneys:

Gomer W. Walters, for appellant.

Judge:

Before Bennett, Henon and Spencer, Examiners-in-Chief.

Opinion Text

Opinion By:

Henon, Examiner-in-Chief.

This appeal is from the decision of the examiner rejecting claims 9 through 19, which constitute all the claims

remaining in the application.

The invention relates to an auger type mixing apparatus for mixing cementitious materials employing a volatile liquid. Representative claim 9 reads as follows:

> 9. Apparatus mounted on a vehicle for mixing a cementitious material in which a volatile liquid is employed comprising:
>
> an enclosed mixing chamber sealed to prevent the escape of the volatile liquid and any potentially dangerous fumes;
>
> a solid frame forming the top of said mixing chamer and having an inlet end thereof pivotably mounted on the vehicle;
>
> an easily removable elastomeric trough forming the bottom of said mixing chamber, the elastomeric material selected to be compatible with the materials being mixed;
>
> an auger having a central shaft and mounted in said frame to convey materials through said mixing chamber;
>
> mixing paddles mounted on the shaft of said auger;
>
> a drive motor for said auger mounted on said frame;
>
> a releasable flexible coupling between the aligned shafts of said motor and said auger to permit removal of said auger from said frame;
>
> an inlet hopper to introduce substantially dry materials into said mixing chamber;
>
> liquid injection means to introduce a liquid into said mixing chamber at a distance removed from said inlet hopper to have said substantially dry material form a plug to prevent the liquid and any fumes from backing up said inlet hopper; and
>
> a discharge opening formed in said elastomeric trough.

The references relied on by the examiner are:

Table set at this point is not available. See table in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.

Claims 9 through 14 and 17 stand rejected as being directed to obvious subject matter within the meaning of 35 U.S.C. 103 in light of the teachings of Zimmerman in view of Wilkinson, Futty, Lasar, Clemens and Cunningham. The examiner contends that Zimmerman discloses the claimed subject matter except for "having the mixing chamber enclosed with a solid top frame and having a removable auger and having liquid injection means and aligned shafts between the motor and auger and a discharge formed in the elastomeric trough," (final rejection, page 2, paper number 5). The examiner cites Wilkinson as disclosing an enclosed mixing chamber

where the enclosure comprises an inverted substantially U-shaped top frame portion and concludes that it therefore would be obvious to the artisan to modify the open frame in Zimmerman to be an enclosed mixing chamber as taught by Wilkinson "if desired." Since Wilkinson also discloses the concept of providing liquid injection means for the introduction of liquid into a mixing chamber remote from the inlet hopper, the examiner concludes that it would therefore be obvious to modify Zimmerman accordingly. Since Lasar discloses the concept of having an auger with mixing paddles mounted thereon wherein the auger is releasably coupled to a frame, the examiner concludes that it would have been obvious to the artisan to modify the auger in Zimmerman as taught by Lasar. Futty is cited to show that it is well known to provide coaxial alignment between an auger shaft and the shaft of a driving motor. Clemens is cited as disclosing the concept of haivng a discharge opening in a trough. The examiner concludes that it would have been obvious in light of Futty and Clemens to modify the auger motor alignment and discharge opening of Zimmerman to be of the nature suggested by Futty and Clemens.

Cunningham is cited as disclosing seal means to preclude leakage of the material within the mixing chamber. The examiner concludes that it would have been obvious in light of the teachings of Cunningham to employ seal means on the modified device of Zimmerman.

Claim 15 stands rejected as being directed to obvious subject matter under 35 U.S.C. 103 in light of the combined teachings of Zimmerman, Wilkinson, Futty, Lasar, Clemens, Cunningham and August. Combining the teachings of Zimmerman, Wilkinson, Futty, Lasar, Clemens and Cunningham in the manner specified supra, the examiner concludes that it would have been further obvious to the artisan in light of the teachings of August to provide spray elements with selectively activated controls since August teaches such devices to be known.

Claims 16, 18 and 19 stand rejected as being directed to obvious subject matter under 35 U.S.C. 103 in light of the combined teachings of Zimmerman, Wilkinson, Futty, Lasar, Clemens, Cunningham and Tiemersma. Combining the teachings of Zimmerman, Wilkinson, Futty, Lasar, Clemens and Cunningham in the manner specified supra, the examiner concludes that it would have been obvious to further modify the structure of Zimmerman to include a gas-filled bearing housing for sealing purposes.

Rather than reiterate the arguments of appellant and the examiner, reference is made to the brief and answer for the respective details thereof.

Opinion

We will not sustain any of the rejections.

[1] Presuming arguendo that the references show the elements or concepts urged by the examiner, the examiner has presented no line of reasoning, and we know of none, as to why the artisan viewing only the collective teachings of the references would have found it obvious to selectively pick and choose various elements and/or concepts from the several references relied on to arrive at the claimed invention. In the instant application, the examiner has done little more than cite references to show that one or more elements or subcombinations thereof, when each is viewed in a vacuum, is known. The claimed invention, however, is clearly directed to a combination of elements. That is to say, appellant does not claim that he has invented one or more new elements but has presented claims to a new combination of elements. To support the conclusion that the claimed combination is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed combination or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references. We find nothing in the references that would expressly or impliedly teach or suggest the modifications urged by the examiner. Additionally, as aforementioned, we find no line of reasoning in the answer, and we know of none, as to why the artisan would have found the modifications urged by the examiner to have been obvious. Based upon the record before us, we are convinced that the artisan would not have found it obvious to selectively pick and choose elements or concepts from the various references so as to arrive at the claimed invention without using the claims as a guide. It is to be noted that simplicity and hindsight are not proper criteria for resolving the issue of obviousness. Note In re Horn, 203 USPQ 969, 971 (CCPA 1979). Accordingly, we will not sustain any of the rejections presented.

The decision of the examiner rejecting claims 9 through 19 as being directed to obvious subject matter within the meaning of 35 U.S.C. 103 is reversed.

- End of Case -

Intellectual Property Library ISSN 1526-8535

In re Hoch

(CCPA)
166 USPQ 406
Decided July 30, 1970
No. 8323
U.S. Court of Customs and Patent Appeals

Headnotes

PATENTS

1. Pleading and practice in Patent Office - Rejections (§ 54.7)

Where reference is relied on to support a rejection, whether or not in a minor capacity, there would appear to be no excuse for not positively including reference in statement of the rejection.

2. Patentability - Composition of matter (§ 51.30)

To overcome prima facie case of obviousness, applicant's proof is insufficient where it relates merely to unexpected properties of compound, rather than to unexpected differences in properties, i. e., actual differences in properties of prior art compounds and properties of claimed compounds; actual differences in properties are required to overcome prima facie case of obviousness because prima facie case, at least to a major extent, is based on expectation that compounds which are very similar in structure will have similar properties; therefore, it must be shown that expectation is unsound - as by showing that there are substantial, actual differences in properties.

Particular patents-Chloride

Hoch, Novel Chlorinated Benzoyl Chloride and Derivatives Thereof and Methods of Preparing Same, claims 3 to 6 of application refused.

Case History and Disposition:

Appeal from Board of Appeals of the Patent Office.

Application for patent of Paul E. Hoch, Serial No. 233,885, filed Oct. 29, 1962; Patent Office Group 120. From decision rejecting claims 3 to 6, applicant appeals. Affirmed.

Attorneys:

Raymond F. Kramer, Buffalo, N. Y., and Donald C. Studley and William J. Schramm, both of Niagara Falls, N. Y., for appellant.

Joseph Schimmel (Jack E. Armore of counsel) for Commissioner of Patents.

Judge:

Before Rich, Almond, Baldwin, and Lane, Associate Judges, and Fisher, Chief Judge, Eastern District of Texas, sitting by designation.

Opinion Text

Opinion By:

Rich, Judge.

This appeal is from the decision of the Patent Office Board of Appeals [1] affirming the rejection of claims 3-6 of application serial No. 233,885, filed October 29, 1962, entitled "Novel Chlorinated Benzoyl Chloride and Derivatives Thereof and Methods of Preparing Same." [2] We affirm.

Claim 3 is drawn to a genus of three compounds-4,5,6,a,a,a - hexachloro - 3 - toluic acid and the corresponding acid chloride and anilide-which have the following structural formulae:

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Claims 4-6 are species claims drawn to the acid chloride, acid, and anilide, respectively. The usefulness of these compounds as herbicides is emphasized by appellant.

The references expressly relied on to support the rejection are:

Molotsky 2,946,817 July 26, 1960
French Patent 820,696 Aug. 2, 1937

Claims 3 and 5 are rejected as unpatentable over Molotsky and claims 3, 4, and 6 as unpatentable over the French patent, both rejections having 35 U.S.C. 103 as their statutory basis. [3]

Molotsky discloses a group of hexachlorotoluene derivatives having the following formula:

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> wherein R_1 is independently selected from the group consisting of hydrogen, aliphatic, cycloaliphatic and aryl radicals, and R_2 is independently selected from the group consisting of aliphatic, cycloaliphatic and aryl radicals.

Molotsky states generally that these compounds are useful

> as intermediate chemicals, as insecticides, in resin production, as fungicides, as vulcanizing agents, as accelerators for the vulcanization of rubber, and as solvents.

Of the compounds specifically disclosed by Molotsky the one structurally most similar to those claimed by appellant is the ethyl ester of 4,5,6,a,a,a-hexachloro-3-toluic acid which differs in structure from appellant's acid, supra, only in that the former has $-COOC_2H_5$ and the latter -COOH in the 3-position. Thus, Molotsky's compound is the ethyl ester of appellant's free acid. Molotsky also discloses 2,3-dicarboxy-4,5,6,a,a,a-hexachlorotoluene which is a free acid differing from appellant's acid only in having carboxyl groups in both the 2- and 3-positions, rather than just the 3-position.

The French patent discloses compounds which the board characterized as "analogs of the claimed anilide and acid chloride" (emphasis added), the claimed compounds differing therefrom in that they contain two chlorine atoms in 4- and 5-positions whereas the French patent compounds do not. With respect to the utility of the disclosed compounds, the French patent states:

> The chlorides of benzene carboxylic acids containing trichloromethyl groups prepared according to the present invention behave as true acid chlorides: they yield esters, anilides, etc., and are important intermediate products in the preparation of dyes, as well as means for the extermination of noxious animals, *treatment of plant diseases,* etc. [Emphasis added.]

The examiner was of the opinion that given Molotsky's disclosure of the ethyl *ester* of 4,5,6,a,a,a-hexachloro-3-toluic acid "the concept of the acid would be obvious in the absence of unobvious properties not possessed by the ester * * *." The board agreed, adding that Molotsky discloses both *acid* and *ester* derivatives of 4,5,6,a,a,a-hexachlorotoluene (although not the specific acid claimed by appellant).

Taking a similar position with respect to the French patent, the examiner noted that the compounds disclosed in this reference and the anilide and acid chloride claimed here differ only in the presence of two additional chlorines in the latter pair and that the compounds of the patent are disclosed as being useful for "the treatment of plant diseases." The board added that appellant did not appear to challenge the prima facie obviousness of the claimed structures and noted that the French patent suggests the possibility of *poly* chlorination of the disclosed compounds.

Appellant argues that, notwithstanding Molotsky's utility disclosure quoted above, this reference does not teach which of the several utilities mentioned is possessed by the ethyl ester in question or whether all are possessed by it. He also argues that mention of "treatment of plant diseases" in the utility disclosure of the French patent shows that the compounds disclosed in this reference

> have a utility opposite to that of the utility possessed by appellant's compounds; whereas they cure plant diseases, appellant's compounds kill plants.

At the outset, we note that appellant has not attempted to explain what the phrase "treatment of plant diseases" would imply to one skilled in the art. He has merely *asserted* that such use is "contradistinguished" from and "antithetical" to the herbicidal use of the claimed compounds. He has in no way supported his suggestion that compounds useful for the "treatment of plant diseases" (whatever that means) would be expected, because of that usefulness, *not* to be useful as herbicides when applied, perhaps, in greater quantities or to specific plants. On the face of it, "treatment of plant diseases" could mean usefulness in controlling plant-infesting organisms rather than usefulness in obtaining a *direct* beneficial effect on the plant per se.

We are thus not persuaded that herbicidal properties are "contraindicated" by the French patent. [4]

Having considered, on the one hand, the very close structural similarities of the claimed compounds and the reference compounds, the utility disclosures of the references, and the suggestion of polychlorination in the French patent, and, on the other hand, the apparent unobviousness of the utility of the claimed compounds as herbicides, we find that a prima facie case of obviousness has been made out by the examiner.

Appellant attempts to overcome this prima facie case of obviousness by arguing the following:

> Even if said references sufficed to render obvious the structure of appellant's compounds, they, as a matter of law, would not render obvious the compounds themselves (*and all the properties that inhere therein*) under 35 U.S.C. 103; for the herbicidal utility of these compounds is contraindicated by these references. In re Lambooy, 49 CCPA 985, 300 F.2d 950, 133 USPQ 270 (1962) and In re Petering and Fall, 49 CCPA 993, 301 F.2d 676, 133 USPQ 275 (1962), cited with approval in In re Papesch, 50 CCPA 1084, 315 F.2d 381, 137 USPQ 43 (1963); In re Huellmantel, 51 CCPA 845, 324 F.2d 998, 139 USPQ 496 (1963); In re Luvisi and Nohejl, 52 CCPA 1063, 342 F.2d 102, 144 USPQ 646 (1965); and In re Ruschig, 52 CCPA 1238, 343 F.2d 965, 145 USPQ 274 (1965). [Emphasis added.]

[2] Reflection on this contention shows it to be appellant's position that if his compounds possess an advantageous *property* which is unobvious (unexpected) in view of the disclosures of the prior art references, then the prima facie case of obviousness necessarily has been overcome and his *compounds* must be held to be unobvious. None of the cited cases, however, explicitly or implicitly supports this proposition. In each, a prima facie case of obviousness was conceded or held to have been established and applicant's proofs, submitted to overcome that prima facie case, related not merely to *unexpected* properties, but rather to unexpected *differences* in properties, [5] i.e., to *actual* differences in the properties of the prior art compounds and the properties of the compounds involved in the appealed claims. Such actual differences in properties are required to overcome a prima facie case of obviousness because the prima facie case, at least to a major extent, is based on the expectation that compounds which are very similar in structure will have similar properties. Therefore, to *overcome* the prima facie case, it must be shown that the expectation on which it is based was in fact unsound- *as* by showing that there are substantial, actual differences in properties.

Inasmuch as the record here is silent as to how the reference compounds and the claimed compounds actually differ in properties, *if at all,* appellant has failed to overcome the examiner's prima facie case of obviousness. [6]

The decision of the board is *affirmed.*

Footnotes

Footnote 1. Consisting of Asp, Lidoff, and Stone, Examiners-in-Chief, opinion by Asp.

Footnote 2. Although this application is stated to be "a continuation in part of * * * application SN 818,517, filed June 8, 1959" (not of record here), it does not appear that appellant has ever contended that the continuation-in-part is entitled to the filing date of the parent insofar as the subject matter of the appealed claims is concerned.

Footnote 3. [1] There are two other cited references:

Newcomer 3,014,965 Dec. 26, 1961 (filed Apr. 29, 1955)
Newcomer 3,253,900 May 31, 1966 (filed May 15, 1961).

Appellant complains that although neither of these patents is mentioned in the statement of either of the appealed rejections and although this fact was pointed out in appellant's brief below, the board approved of their use by the examiner "as suggesting that [appellant's] compounds would exert herbicidal action" and characterizing

this as a use in a " *minor* capacity" (emphasis added) to "further support the rejection." Appellant's complaint seems to be justified, and if we did not find the rejections based *solely* on Molotsky and the French patent to be sound, we might well feel constrained to reverse the decision of the board. Where a reference is relied on to support a rejection, whether or not in a "minor capacity," there would appear to be no excuse for not positively including the reference in the statement of rejection.

Footnote 4. This, of course, is not to say that herbicidal properties *are* indicated by the French patent either.

Footnote 5. As used in these cases, the meaning of "differences in properties" has included *significant* differences in degree of the *same* property amounting to marked superiority.

Footnote 6. It should be noted, however, that in considering whether a prima facie case of obviousness had been made out we did take into account, inter alia, not only the differences between the structures of the prior art compounds and those claimed, but also the differences in the properties disclosed in the prior art to be possessed by the former and the properties which appellant found to be possessed by the latter, the latter properties being considered notwithstanding appellant's failure to establish actual differences in properties.

- End of Case -

706.02(j) Contents of a 35 U.S.C. 103 Rejection

35 U.S.C. 103 authorizes a rejection where, to meet the claim, it is necessary to modify a single reference or to combine it with one or more other references. After indicating that the rejection is under 35 U.S.C. 103, the examiner should set forth in the Office action:

(A) the relevant teachings of the prior art relied upon, preferably with reference to the relevant column or page number(s) and line number(s) where appropriate,

(B) the difference or differences in the claim over the applied reference(s),

(C) the proposed modification of the applied reference(s) necessary to arrive at the claimed subject matter, and

(D) an explanation why one of ordinary skill in the art at the time the invention was made would have been motivated to make the proposed modification.

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure. *In re Vaeck,* 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). See MPEP § 2143 - § 2143.03 for decisions pertinent to each of these criteria.

The initial burden is on the examiner to provide some suggestion of the desirability of doing what the inventor has done. "To support the conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references." *Ex parte Clapp*, 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985). See MPEP § 2144 - § 2144.09 for examples of reasoning supporting obviousness rejections.

Where a reference is relied on to support a rejection, whether or not in a minor capacity, that reference should be positively included in the statement of the rejection. See *In re Hoch,* 428 F.2d 1341, 1342 n.3 166 USPQ 406, 407 n. 3 (CCPA 1970).

It is important for an examiner to properly communicate the basis for a rejection so that the issues can be identified early and the applicant can be given fair opportunity to reply. Furthermore, if an initially rejected application issues as a patent, the rationale behind an earlier rejection may be important in interpreting the scope of the patent claims. Since issued patents are presumed valid (35 U.S.C. 282) and constitute a property right (35 U.S.C. 261), the written record must be clear as to the basis for the grant. Since patent examiners cannot normally be compelled to testify in legal proceedings regarding their mental processes (see MPEP § 1701.01), it is important that the written record clearly explain the rationale for decisions made during prosecution of the application.

See MPEP § 2141 - § 2144.09 generally for guidance on patentability determinations under 35 U.S.C. 103, including a discussion of the requirements of *Graham v. John Deere*, 383 U.S. 1, 148 USPQ 459 (1966). See MPEP § 2145 for consideration of applicant's rebuttal arguments. See MPEP § 706.02(l) - § 706.02(l)(3) for a discussion of prior art disqualified under 35 U.S.C. 103(c).

706.02(k) Provisional Rejection (Obviousness) Under 35 U.S.C. 102(e)/103 [R-2]

Effective November 29, 1999, subject matter which was prior art under former 35 U.S.C. 103 via 35 U.S.C. 102(e) is now disqualified as prior art against the claimed invention if that subject matter and the claimed invention "were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person." This change to 35 U.S.C. 103(c) applies to all utility, design and plant patent applications filed on or after November 29, 1999, including continuing applications filed under 37 CFR 1.53(b), continued prosecution applications filed under 37 CFR 1.53(d),